Lecture Supplements

Plant Bacteriology

Bacterial Disease Management-Part 2

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The second defense line:

- **Oxidative burst**
- **SAR, LAR,..**

Active Defense Mechanisms

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- The oxidative burst (ROIs)
- The generation and role of nitric oxide (NO) in resistance
- Callose synthesis and deposition

2. Slower responses implicated in resistance

- The hypersensitive response
- Phytoalexins
- Lignification
- Suberization
- Synthesis of hydroxyproline-rich glycoproteins (HRGPs)
- Pathogenesis-related proteins (PRPs)
- Systemic acquired resistance (SAR)
- Induced systemic resistance (ISR)

3. Elicitors of defense responses

4. Elicitor perception

5. Signalling

Increases in cytosolic calcium
Mitogen-activated protein kinases (MAP kinases)
Salicylic acid (SA)
Jasmonates
Ethylene
Integration of signalling pathways

Active Defence Mechanisms

Major mechanisms

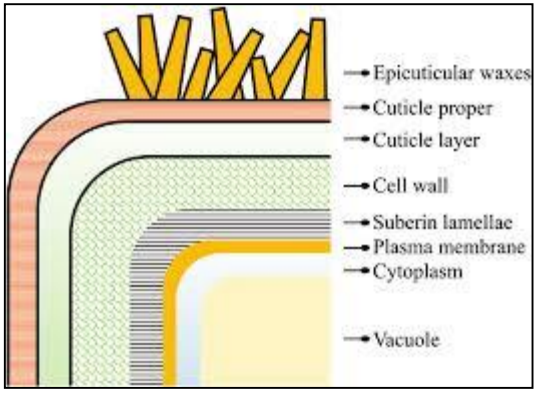
1. The hypersensitive response
2. Phytoalexin synthesis
3. Lignification
4. The synthesis of pathogenesis-related proteins,
5. Hydroxyproline-rich glycoproteins, and
6. Systemic acquired resistance (SAR).

- All these require gene transcription and protein synthesis.

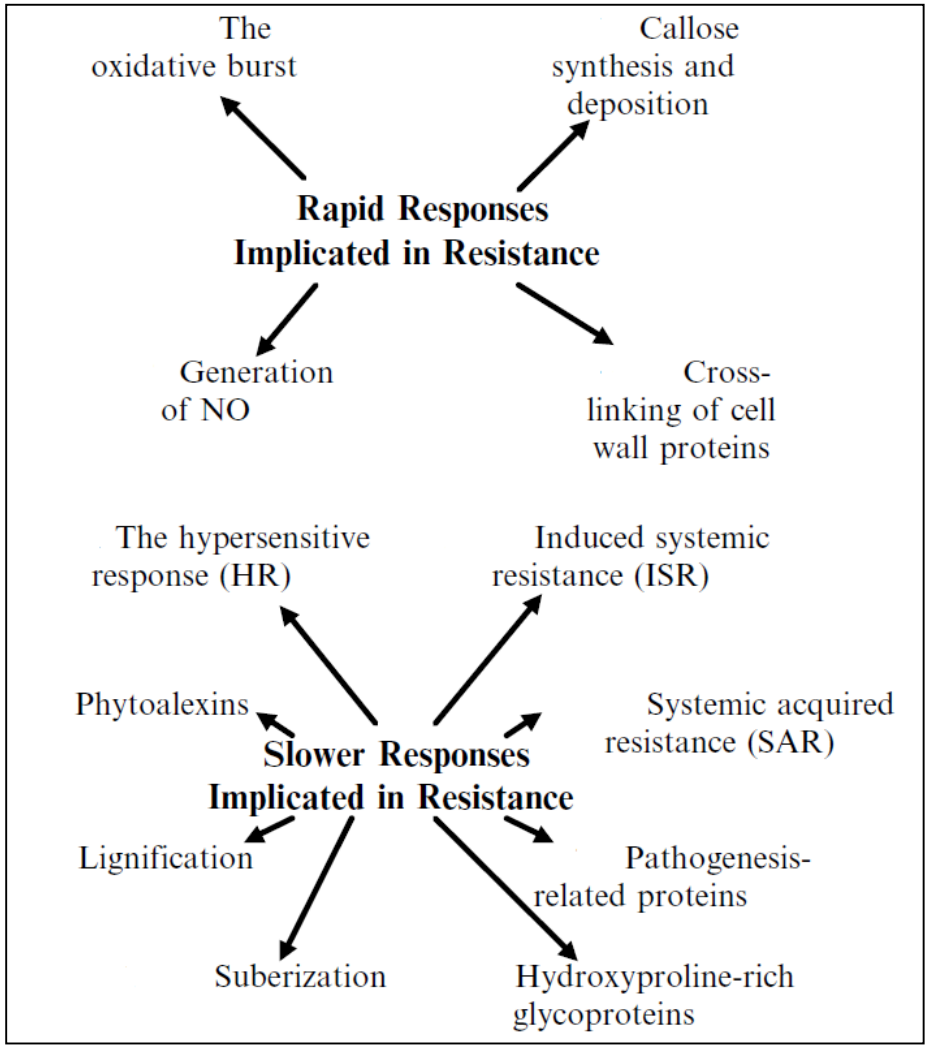
Strange,2003

Active defence mechanisms of plants
Summarized diagrammatically

Suberin is a complex polyester built from poly-functional long-chain fatty acids (suberin acids) and glycerol. Suberin is a lipophilic macromolecule found in specialized plant cell walls, wherever insulation or protection toward the surroundings is needed.
Nitric oxide (nitrogen oxide or nitrogen monoxide) is a colorless gas with the formula NO.



Strange,2003;..



Active Defense Mechanisms

1. Oxidative burst and nitric oxide (NO)

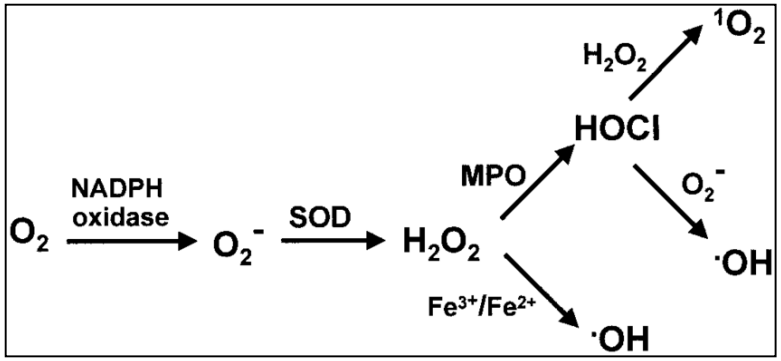
- Reactive oxygen intermediates (ROIs) protect the plant in several ways:

1. They are toxic to microorganisms. This toxicity is thought to be potentiated by NO.
   - In fact ROIs with NO cause the hypersensitive death of host cells (HR).
2. Hydrogen peroxide acts as a substrate for lignification, making cell walls more difficult to penetrate.
   - It is also involved in the induction of protective genes coding for pathogenesis-related proteins.

Active Defense Mechanisms

Oxidative burst and reactive oxygen intermediates (ROIs)

- During the oxidative burst, reactive oxygen intermediates (ROIs) are generated which include:
- The superoxide anion (O_2),
- Hydrogen peroxide (H_2O_2), and
- The hydroxyl radical (OH).



NADPH oxidase constitutes a family of enzymes whose function is to catalyze the transfer of electrons to O_2 generating superoxide or H_2O_2 using NADPH (nicotinamide adenine dinucleotide phosphate oxidase) as an electron donor; SOD (superoxide dismutase), MPO (myeloperoxidase).

Active Defense Mechanisms

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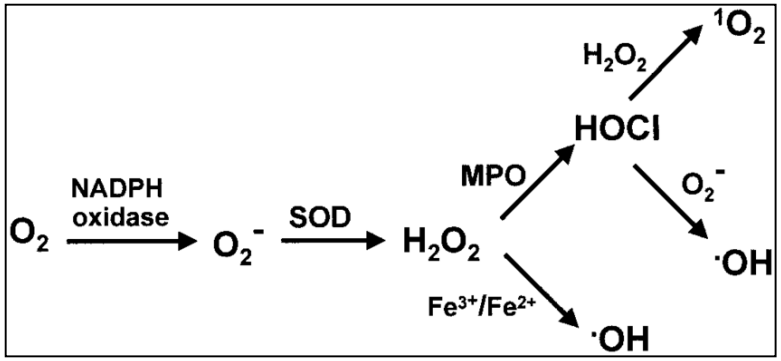

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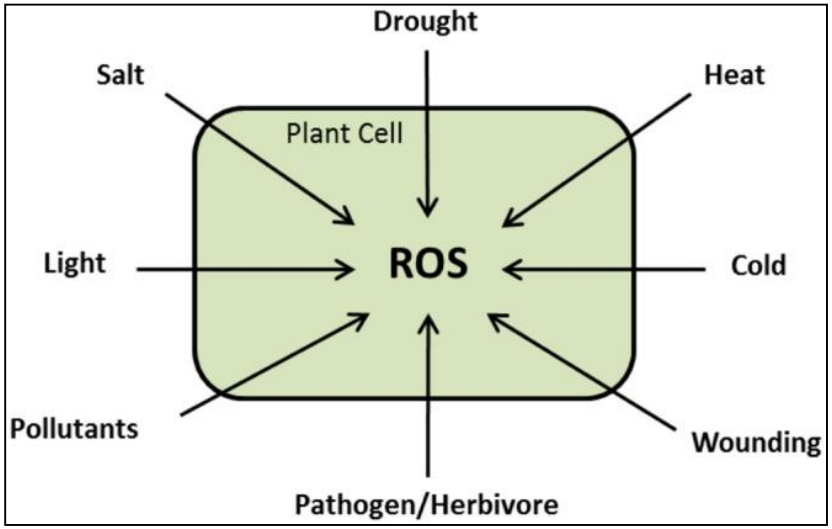
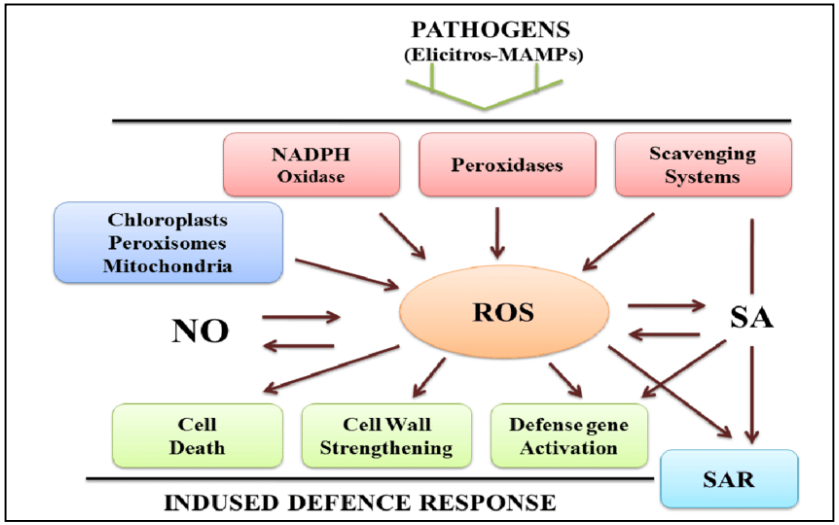
Reactive oxygen intermediates and nitric oxide

ROI and NO



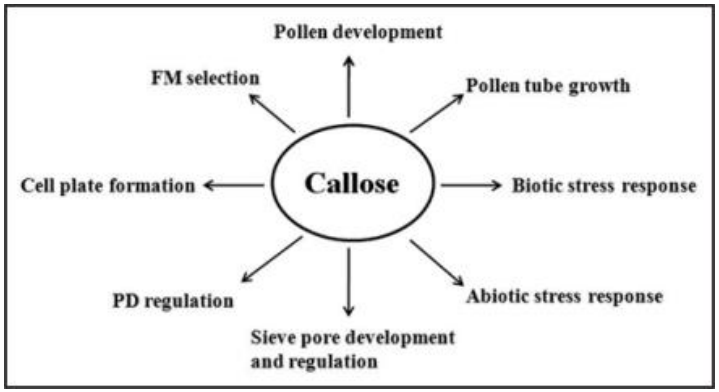


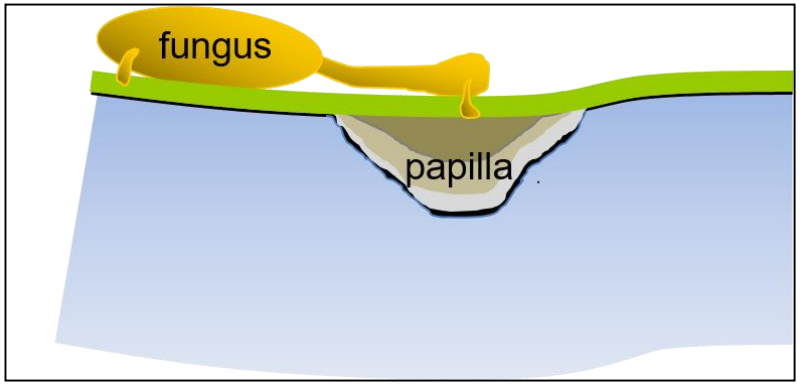
ADPH is used in anabolic reactions, such as lipid and nucleic acid synthesis, which require NADPH as a reducing agent. Nitric oxide (NO) as a colorless gas serves as signal in hormonal and defense responses.

Active Defense Mechanisms

2. Callose synthesis and deposition as papillae

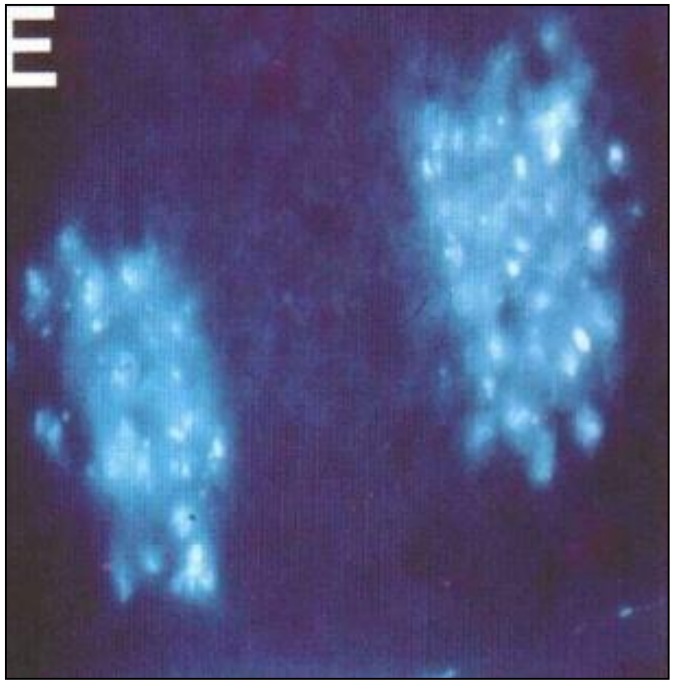
- Callose is a β-1,3-linked glucan which is often synthesized and deposited as papillae- localized wall appositions as an early response to wounding or pathogen attack.
- The speed of callose deposition was emphasized in work with French bean (*Phaseolus vulgaris*) and *Xanthomonas campestris*.





Callose deposition

- Callose deposition in Arabidopsis leaf mesophyll at the incompatible infection sites.
- Callose was detected by aniline blue staining and fluorescence under UV light (Parker *et al.*,1993).



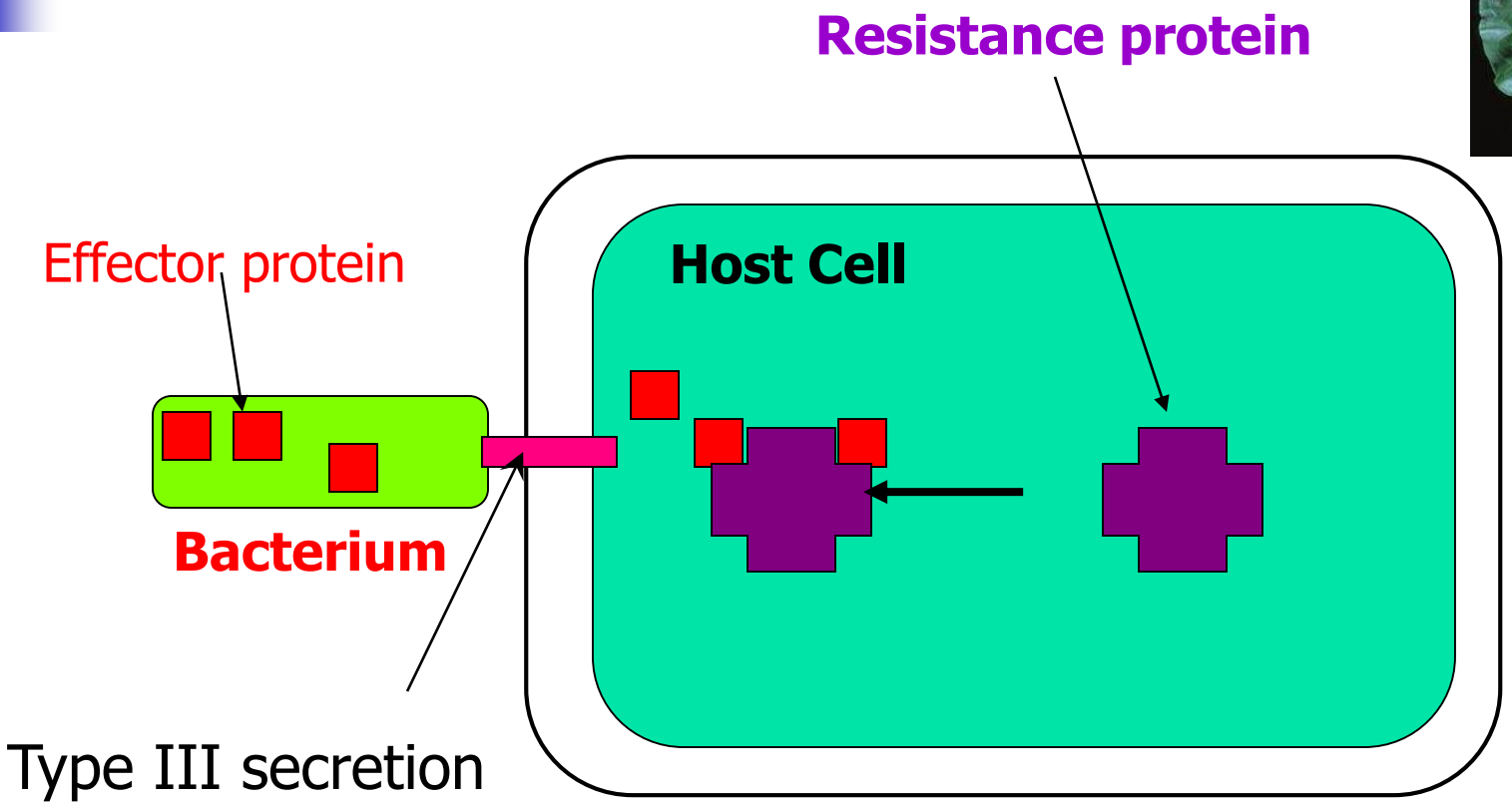
Active Defense Mechanisms

3. The hypersensitive response

- The hypersensitive response (HR) is an essentially universal reaction of plants challenged with avirulent pathogens.
- Challenged cells and sometimes those in their immediate vicinity die rapidly (i.e. react hypersensitively) and this limited necrosis is associated with resistance of the plant as a whole.

Active Defense Mechanisms

The hypersensitive response

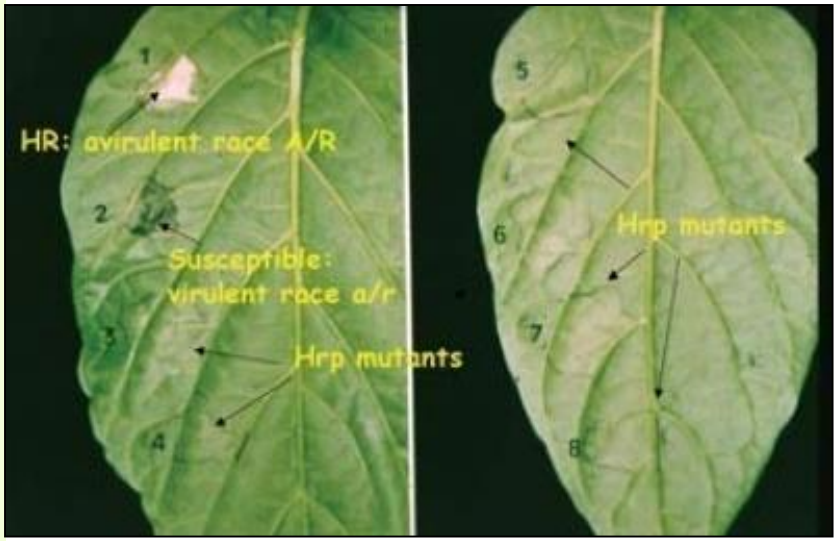


Reactions of pepper to *Xanthomonas vesicatoria*

Virulence, avirulence and the null reaction to Hrp mutants

- The water-soaked lesions of the areas of the leaf inoculated with the virulent isolate a/r and compare with:

1. The hypersensitive cell death caused by the avirulent race A/R.
2. The Hrp mutants (3-8) are deficient in the type III secretory system and therefore cannot export effector molecules that cause either the susceptible reaction or the hypersensitive response.



Interaction	Pathogen	Plant Host
Compatible ↓ Disease	Virulent	Susceptible (Tomato)
Incompatible ↓ HR	Avirulent	Resistant (Tobacco)

Strange,2003

Phytoalexins

Large differences in sensitivity of the pathogens

- Phytoalexins are inducible antimicrobial metabolites in plants.
- Phytoalexins (Phyto= plant and alexin = to ward off).
- Low molecular mass antimicrobial compounds that are produced by plants as a response to biotic and abiotic stresses.
- Many phytoalexins have been isolated from plants (>20 families).
- E.g. Leguminosae, Solanaceae, Malvaceae, Graminae, Compositae, Umbelliferae, Chenopodiaceae.

Phytoalexins
Induced antimicrobials

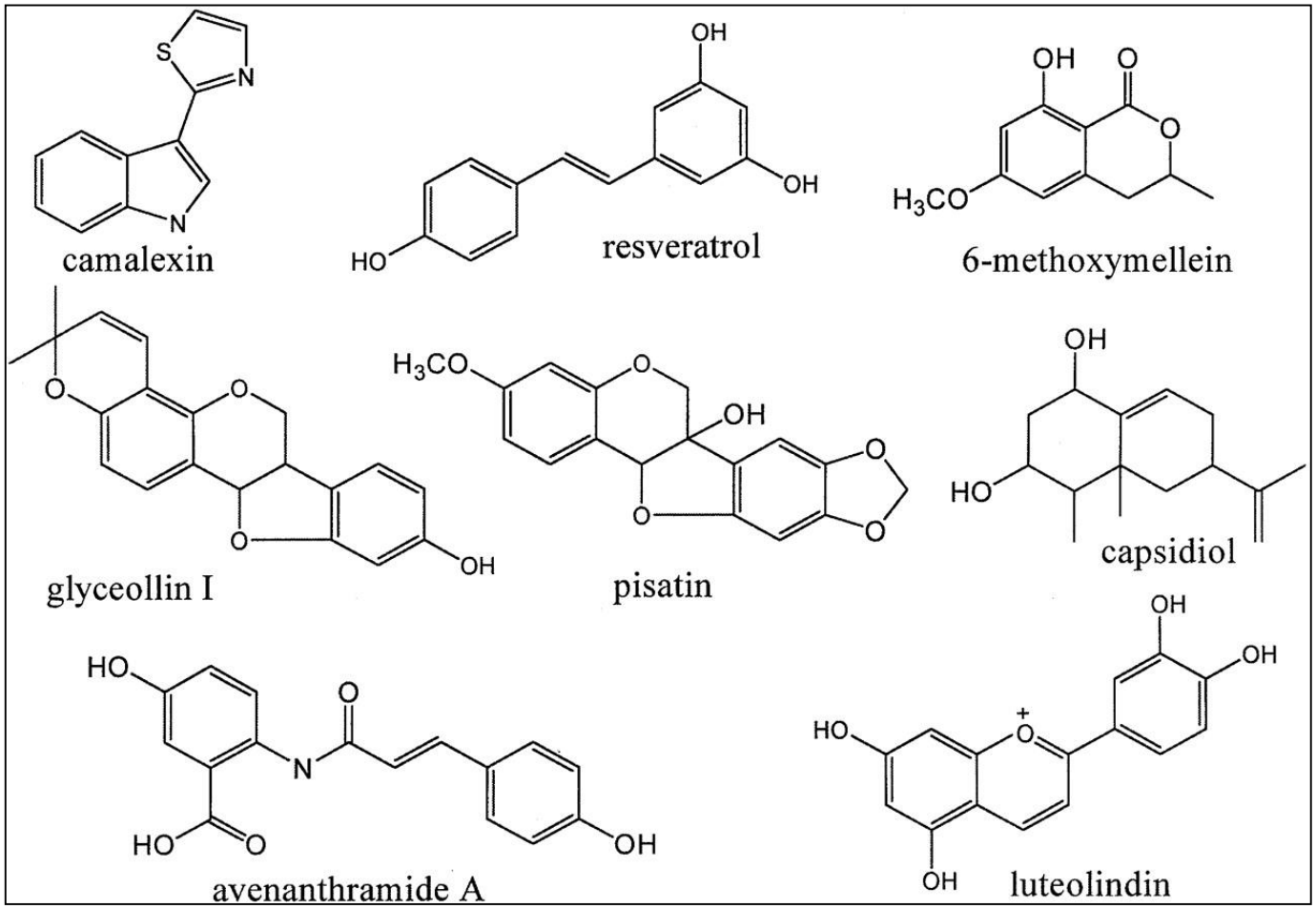
- Low molecular weight, accumulate after pathogen infection.
- Chemically diverse (i.e. not all the same!)
- Non-specific biocides, affecting a wide range of organisms including bacteria, fungi, nematodes, higher animals and plants themselves and some of those from legumes have the added property of being estrogenic.

Phytoalexins are antimicrobial and often antioxidative substances synthesized *de novo* by plants that accumulate rapidly at areas of pathogen infection. They are broad spectrum inhibitors and are chemically diverse with different types characteristic of particular plant species.

Wikipedia,2021

Phytoalexins

Selected phytoalexin structures



Hammerschmidt,1999

Phytoalexins

Large differences in sensitivity of the pathogens

- Gram-positive bacteria being more sensitive than Gram-negative bacteria.

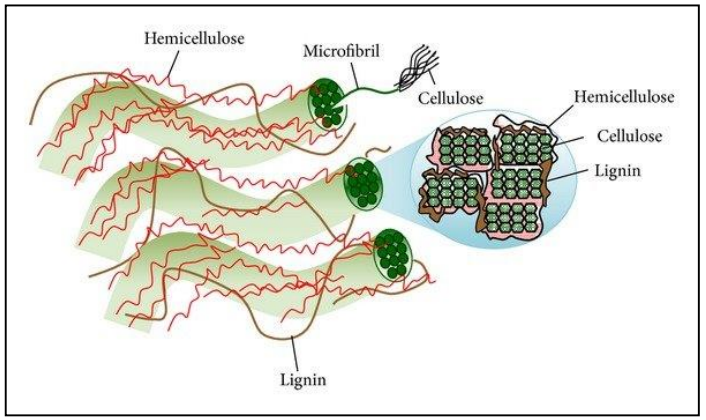
1. Large differences in sensitivity, however, may be found among pathogens which attack plants that accumulate phytoalexins.
2. Virulent strains usually tolerating higher concentrations than avirulent ones.

- Such differences are usually attributable to the superior ability of virulent strains to degrade the phytoalexin.

Active Defense Mechanisms

4. Lignification

- Lignin is a very complex and resistant structure and lignification is thought to contribute to resistance by increasing the mechanical force required for penetration, increasing the resistance of cell walls to degradation by enzymes of the pathogen and setting up impermeability barriers to the flow of nutrients and toxins.
- Lignin precursors such as coniferyl alcohol and free radicals may be toxic to the pathogen *per se.*

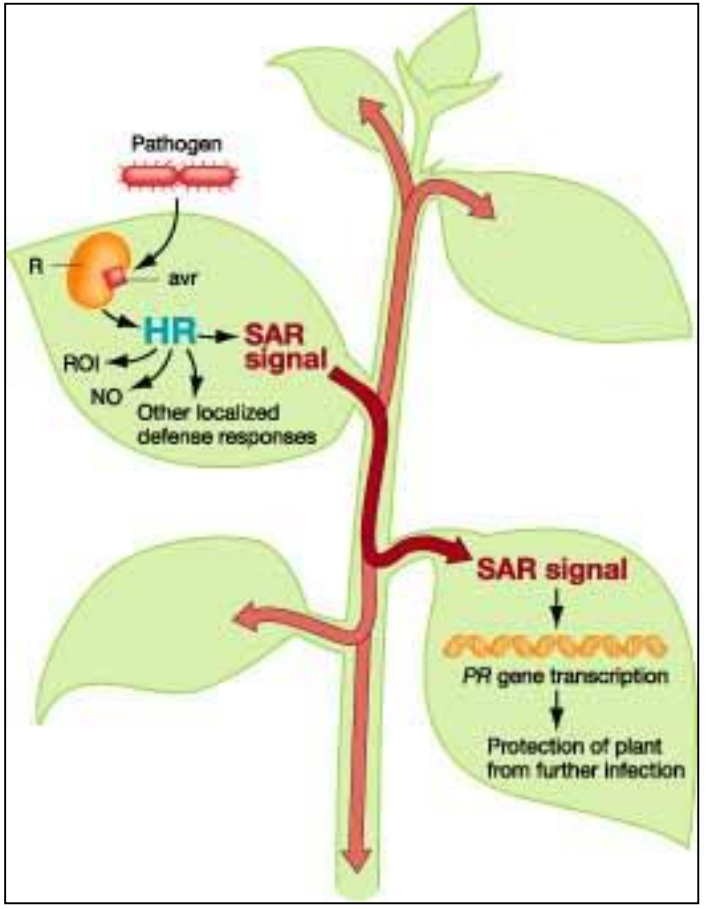


Active Defense Mechanisms

Effector-triggered immunity (ETI)

Induced/systematic resistance

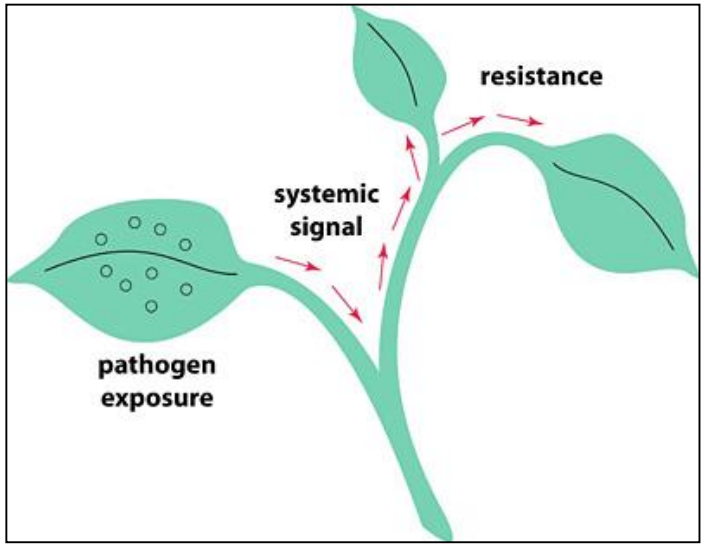
- Systemic acquired resistance (SAR) refers to a distinct signal transduction process, that plays an important role in the ability of plants to defend themselves against pathogens.
- After the formation of a necrotic lesion, either as a part of hypersensitive response (HR) or as a symptom of disease, the SAR process is activated.



Naga Satyasri Ch; Elmore,2015

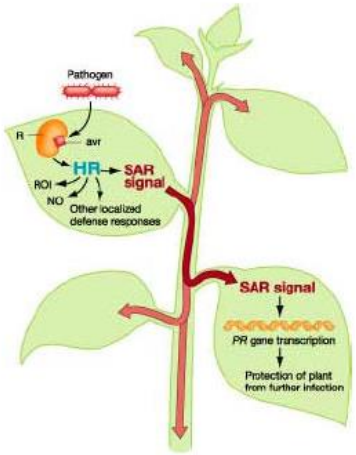
Active Defense Mechanisms

Induced/Systematic Resistance



Nonspecific induced resistance

- Induced by:
  1. Pathogenic challenge
  2. Chemical signals
     - Salicylic acid
     - ACTIGARD®
- Costs to plant
  1. Reduced yield in absence of disease
  2. Suppressed insect-wound response



Microbial genomes sequencing

"Immediate Priority" for diseases control

Microbial genomes sequencing

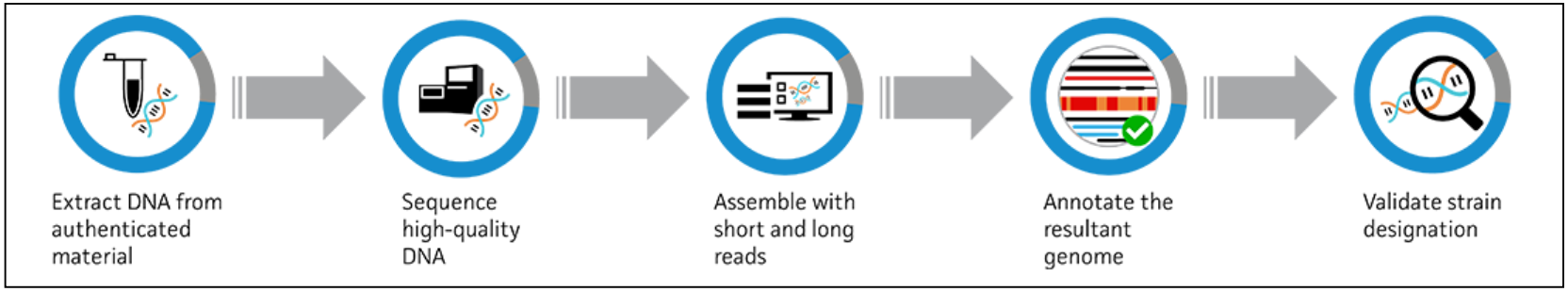
- Analyses of microbial genomes will complement those done on plant genomes (e.g. for *Arabidopsis*, rice, etc.) by providing new insights into the nature of plant-microbe interactions.
- Population genomics studies is a good way to study:

1. the adaptive evolution of plant pathogens, and
2. design better disease management strategies.

Microbial genomes sequencing

Comprehensive ATCC bacterial whole-genome sequencing workflow

- The genome of an organism refers to its entire complement of genes contained in the DNA of its chromosome (s).
- Pathogenic bacteria possess certain features referred to as virulence determinants which enable them to cause disease in susceptible hosts.



ATCC 2021;..

Microbial genomes sequencing

Bacterial genomes vs. genomes of eukaryotes

- Pathogenic bacteria possess certain features referred to as virulence determinants which enable them to cause disease in susceptible hosts.
- genomes are generally smaller and less variant in size among species when compared with genomes of eukaryotes.
- Bacterial genomes can range in size anywhere from about 130 kbp to over 14 Mbp.

Wikipedia,2021; Donkor,2013

Microbial genomes sequencing

Bacterial genomes vs. genomes of eukaryotes

- Since the advent of genome sequencing two decades ago, about 1,800 bacterial genomes have been fully sequenced.
- Nowadays, sequencing the genome of the pathogen has become a routine and first step for plant pathologists. By now (2019), approximately 126 plant pathogenic bacterial species have been sequenced.
- Among them, the largest genome sequenced for plant pathogenic bacteria is 11.5 Mb, whereas the smallest genome sequenced is 600 Kb.

Wikipedia,2021; Xu and Wang,2019

The complete genome sequence for plant pathogenic prokaryotes

PLANT PATHOGENIC BACTERIA AND MOLLICUTES

≤10 - IMMEDIATE PRIORITY SPECIES:
(Alphabetically, arranged - not ranked within the list)

Organism	Strain	Genome Size	Rationale /Significance
Burkholderia cepacia	ATCC 25416	7.9 Mb incl 3.5, 3.1, 1.1, and 0.2 Mb replicons	Causes sour skin of onion, cavity disease of mushroom, brown spot of orchids, and rot of caladium bulbs. Strain ATCC 25416 is a plant-pathogen representative of genomovar I strains, which have rarely been isolated as human pathogens. Opportunity for direct genome comparison to genomovar III strains, which are more typically associated with cystic fibrosis patients exhibiting symptoms of Cepacia syndrome.
Clavibacter michiganensis subsp. *insidiosis*	NCPPB 1109	ca. 3.0	Causes bacterial wilt, an economically important disease of alfalfa. Very little known of its molecular biology due to the difficulty in genetic manipulation of the pathogen. Recent advances in the manipulation of related pathogens and the soon-to-be-available genome sequences of *Clavibacter michiganensis* subsp. *sepedonicus*, *C. michiganensis* subsp. *michiganensis* and *Leifsonia xyli* will enable genomic and functional comparisons across phylogenetically-related vascular pathogens.
Clover Phyllody Phytoplasma		ca. 0.8 Mb	Clover phyllody is widespread in North America and Europe where it is responsible for diseases in fruits and vegetables. Host plants damaged by this phytoplasma include *Trifolium* spp., *Fragaria* spp. (strawberry), *Olea* spp. (olive), *Poa pratensis*, *Anemone* spp., *Ranunculus* spp., and *Vitis* (grapevine). This phytoplasma is a member of subgroup C in group I (the aster yellows group) and is a distinct species from aster yellows phytoplasma.
Pantoea citrea	1056R	ca. 5 Mb	Causes pink disease of pineapple, resulting in $130,000,000 loss per annum. Next to mangos (#1) and bananas (#2), pineapple is the third most consumed fruit in the world. Genomics would help us identify novel genes involved in invasion of the fruit, repression of the hypersensitive reaction in the host, induction of genes involved in glucose oxidation which leads to pink coloration, etc. Opportunity for genome-scale comparison with enterobacterial pathogens (virulence factors and evolution of pathogenesis).

The complete genome sequence for plant pathogenic prokaryotes

PROKARYOTIC PLANT PATHOGENS BEING SEQUENCED (additional funding for completion may be needed):

Organism	Strain	Genome Size	Rationale /Significance
Pectobacterium carotovorum subsp. *carotovorum*	Ecc71	ca. 5 Mb	Causes bacterial soft rot and related diseases of numerous crops worldwide, under temperate as well as tropical conditions and under field conditions, in storage and in transit. Opportunity for genome-scale comparison with enterobacterial pathogens (virulence factors and evolution of pathogenesis). Because this pathogen has world-wide economic importance and has been a principal model organism for molecular studies of plant-microbe interactions, the scientific community who would benefit from the genomice sequence is particularly large.
Ralstonia solanacearum Race 3 Biovar 2	UW551	ca. 5.8 Mb	Causes potato brown rot, the third leading source of potato yield losses in the developing world. It is also a virulent pathogen of geraniums and tomatoes, causes latent infections of weeds and native plants, and is a quarantine pest and an agroterrorism Select Agent for the US. It is a model organism for understanding pathogenesis and systemic vascular colonization of host plants. Comparative genomics with *Burkholderia cepacia* and Race 1 of *R. solanacearum*, a model organism for molecular studies, will elucidate host specificity and microevolution. Large, international research community.
Spiroplasma citri	BR3-3X	1.6 Mb	Spiroplasmas are unique bacteria characterized by tiny cell and genome sizes, lack of a cell wall, helical morphology, and an unusual genetic code. *Spiroplasma citri* causes disease in a number of plant species including citrus, and also colonizes its insect vectors. *S. citri* strain BR3-3X, isolated from brittle-root diseased horseradish plants in Illinois, has been the model system for U.S. research on spiroplasma-vector interactions for over 20 years
Xanthomonas axonopodis pv. malvacearum		ca. 5.0-5.2 Mb	Causes bacterial blight, a worldwide problem on cotton resulting in yield losses to 50%, with 10-30% losses occurring commonly. The pathogen has served as a model bacterial gene-for-gene system with the highest level of race specificity of any xanthomonad (over 20 races described). The African strains are of particular interest because they are quarantined pathogens in the U.S., and they are capable of defeating most of the known bacterial blight resistance genes in cotton. The pathogen is amenable to standard microbial genetic tools, and the histology, biochemistry and physiology of the disease have been very well described, giving enormous leverage to the sequence information.

The complete genome sequence for plant pathogenic prokaryotes

PLANT ASSOCIATED BENEFICIAL PROKARYOTES AND EUKARYOTES ≤25 - HIGH PRIORITY SPECIES: (Alphabetically, arranged - not ranked within the list)			
Prokaryotes:			
Bacillus cereus	UW85	ca. 5.2	Common soil inhabitant involved in biological control of plant pathogens. Opportunity for genome comparisons with close relatives for which genome sequences have already been obtained, i.e. *B. anthracis* and a clinical isolate of *B. cereus*.
Burkholderia ambifaria	AMMDR1	ca. 7.2	Common rhizosphere organism. Biocontrol of soilborne oomycetes. Opportunity for genome-scale comparison with human pathogenic strains of the *Burkholderia cepacia* complex.
Pseudomonas fluorescens	A506	ca 5.5	Common plant epiphyte. Commercial biological control strain for fire blight. Opportunity for genome-scale comparison with other fluorescent pseudomonads.
Pseudomonas fluorescens	Q8r1	ca 5.5	Aggressive rhizosphere colonist and biological control agent of root diseases. Type strain for D genotype of DAPG producers. Biovar distinct from *P. fluorescens* Pf-5 which is currently being sequenced. Opportunity for comparisons with other fluorescent pseudomonads.
Pseudomonas aureofaciens	30-84	ca 5.5	Phenazine producer and model strain for genetic studies of biological control and quorum sensing. Opportunity for genome-scale comparison with other fluorescent pseudomonads.

The complete genome sequence for plant pathogenic prokaryotes

PLANT ASSOCIATED BENEFICIAL PROKARYOTES AND EUKARYOTES ≤10 - IMMEDIATE PRIORITY SPECIES: (Alphabetically, arranged - not ranked within the list)			
Organism	**Strain**	**Genome Size Mb**	**Rationale/Significance**
Prokaryotes:			
Bacillus subtilis	GB03	ca. 4.3	One of the most widely distributed bacterial species in agricultural systems. This rhizosphere isolate used in biocontrol of soilborne root diseases. Well-established commercial applications. Excellent opportunity for genome-scale comparisons with non-functional saprophyte and human pathogens of same genera.
Pantoea agglomerans	C9-1	ca 4.0	Common plant epiphyte. Biological control strain for fire blight. Opportunity for genome-scale comparison with enterobacterial pathogens.

Microbial genomes sequencing
Significantly improved our understanding of plant-microbe interaction

- A complete understanding of disease susceptibility and resistance will require understanding:
- The interactions between:

1. plant hosts,
2. pathogen, and
3. beneficial microorganisms.

- Sequence data from the genes for such microorganisms is essential to:

1. advance our knowledge of infection and the interaction of pathogens and host gene products, and will provide knowledge on
2. how these organisms reproduce and spread.

Micr
obial genomes sequencing
Significantly improved our understanding of plant-microbe interaction

- Microbial sequencing is performed for a diverse set of applications, such as:

1. microbial identification and taxonomy,
2. population genetics and meta-transcriptomic.
3. environmental monitoring,
4. pathogen detection,
5. routine testing of materials for bacterial contamination,
6. understanding about the biology of many bacterial pathogens,
7. identification of novel antibiotic targets.

Wikipedia,2021; Donkor,2013

Microbial genomes sequencing
Significantly improved our understanding of plant-microbe interaction

- Bacteria become pathogenic due to the presence of virulence factors, which are often obtained by genetic transfer with other organisms.
- Virulence factors detection capabilities include:

1. Simultaneously aligning large reference databases of known virulence sequences;
2. Identifying genes and/or regulatory sequences associated with virulence;
3. Estimating threat level based on the number and types of pathogenic factors.

Noblis Inc,2019

Microbial genomes sequencing

Significantly improved our understanding of plant-microbe interaction

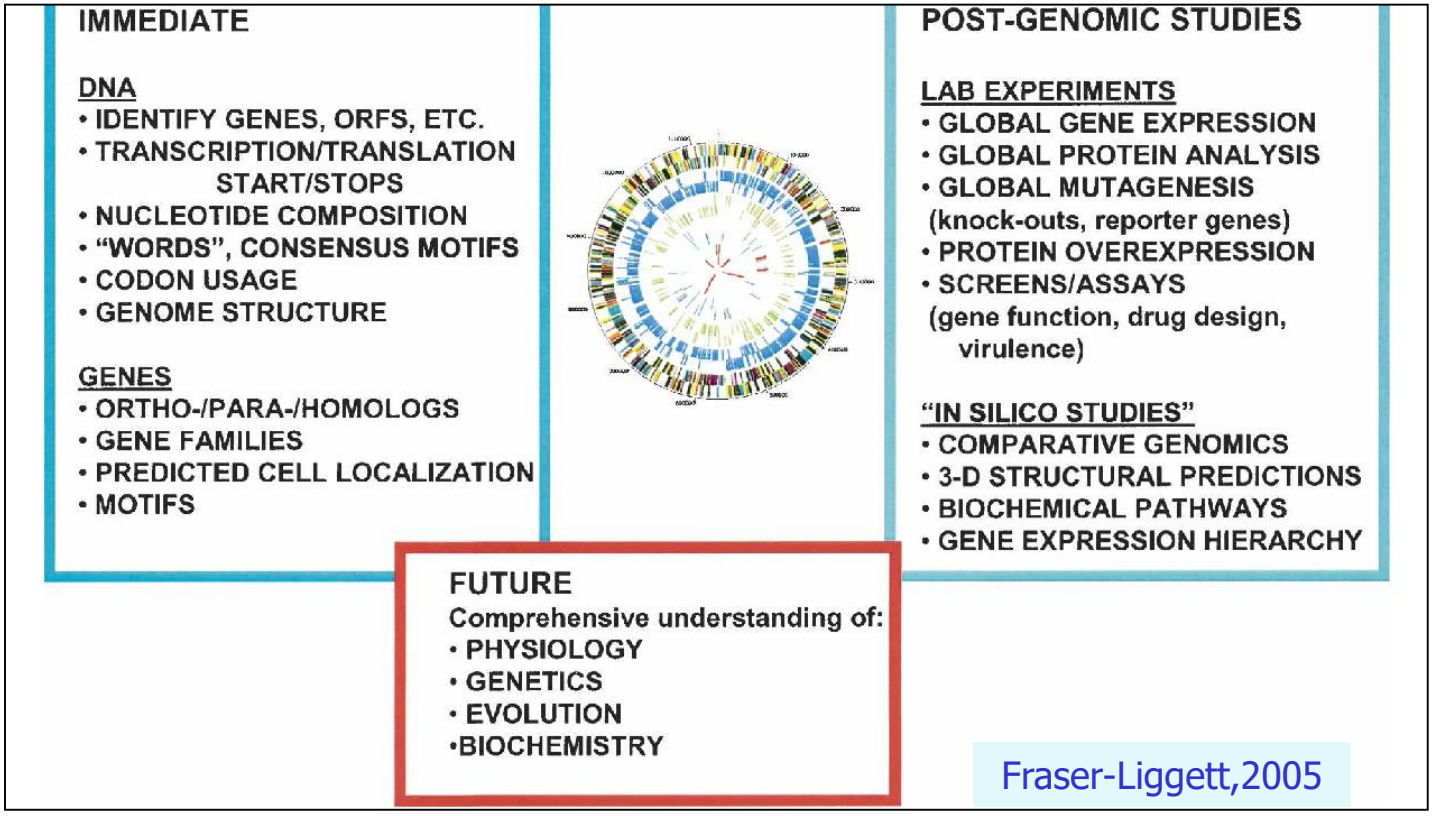
- Genomic analyses of plant associated microorganisms are as essential to understanding the development and suppression of plant diseases.
- Effective control will then be achieved through:

1. developing host resistance, or
2. through means that combat the pathogen directly or indirectly.

- Studies of microorganisms with biocontrol capacities may lead to further development of environmentally safe chemical controls for plant pathogens.

Microbial genomes sequencing

Significantly improved our understanding of plant-microbe interaction



Applications and future directions in microbial genomics. The availability of microbial DNA sequence has provided a new foundation for follow-up studies, both in vitro and in silico. The ultimate goal is to integrate data from these multiple approaches to achieve a new systems-level understanding of the microbial cell.

Microbial genome informations

***avr* genes**

Pathogen-generated secreted proteins
Secretomes

- Investigations of microbial secretomes, e.g., the set of proteins that are secreted by a microbe, have been especially informative in revealing conserved mechanisms of pathogenicity among bacterial plant pathogens.
- Pathogens produce several classes of secreted proteins, some of which function in virulence or plant defense.

The *avr* genes
Bacterial effector

- The term effector refer to pathogen proteins that are presented to the plant cell during infection.
- These proteins probably evolved to facilitate the pathogen life cycle and are thus virulence factors.

Effector diversity

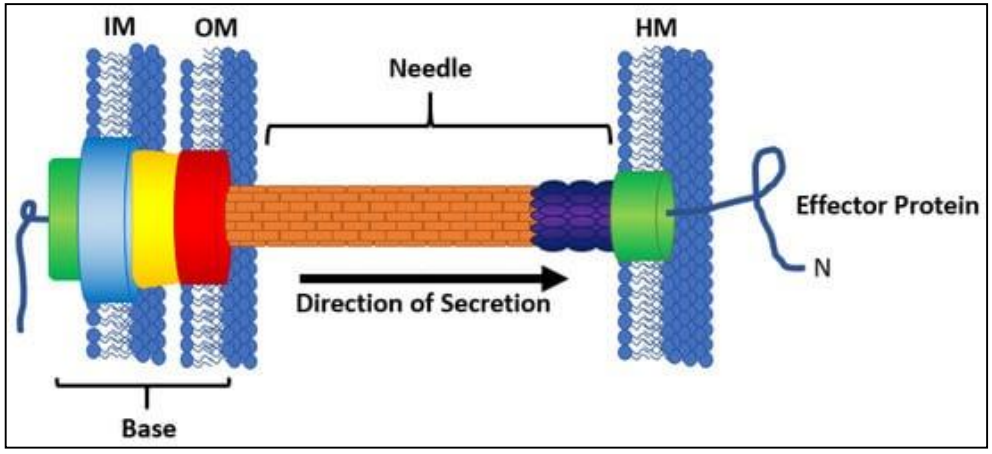
Some with no obvious biochemical activity or phenotypic effects

- Among bacteria, there is a growing list of over 40 diverse effectors (about 50) identified both from screens for proteins that are delivered by the type III secretion system (TTSS).
- Most bacterial effectors have no obvious biochemical activity or phenotypic effects on plants lacking a corresponding R protein.

Effector diversity

Type III secretion system (TTSS)

- The type III systems encoded by *hrp* genes serve to deliver secreted proteins known as effectors into plant cells and these include both *avr* and *vir* gene products.
- Type III secretary system is also required for the secretion of avr proteins.



Pendergrass and May,2020

avr genes

With obvious functions

- So far only one effector gene product of *P. syringae* has been shown to function as an enzyme that is involved in the synthesis of syringolides, which are able to elicit the hypersensitive response.
- Further results indicate that the AvrBs2 effector of *X. campestris* may also be an enzyme and act within plant cells.

avr genes
With obvious functions

- Most Avr proteins are considered virulence factors required for the colonization of host plants.
- Some other avirulence products may be involved in virulence (causing disease in a compatible interaction).
- Some of *avr* genes which play a role in pathogenicity on susceptible plants are:

1. *AvrBs3* TAL effector family from Xanthomonds. E.g. *avrBs2* from *X. vesicatoria*,
2. *avrRpm1* from *P. syringae* pv. *maculicola*, and
3. *pthA* from *X. citri*.

AvrBs3 TAL effectors

TAL effector in *Xanthomonas* spp. and Bs3 resistance gene in host plants

- TAL effectors are important virulence factors of bacterial plant pathogenic Xanthomonas, which infect a wide variety of plants including valuable crops like pepper, rice, and citrus.
- TAL proteins are translocated via the bacterial type III secretion system into host cells and induce transcription of plant genes by binding to target gene promoters.
- Members of the TAL effector family differ mainly in their central domain of tandemly arranged repeats of typically 34 amino acids each with hypervariable di-amino acids at positions 12 and 13.

Scholze and Boch,2010

AvrBs3 TAL effectors

TAL effector in *Xanthomonas* spp. and Bs3 resistance gene in host plants

- Members of the TAL effector family differ mainly in their central domain of tandemly arranged repeats of typically 34 amino acids each with hypervariable di-amino acids at positions 12 and 13.
- AvrBs3, contains 17.5 nearly-identical tandem repeats of 34 amino acids (aa) which differ mainly at positions 12 and 13, termed repeat variable diresidue (RVD).
- The repeat region turned out to be a novel DNA-binding motif of the *Xanthomonas* type III effectors AvrBs3 with **one repeat** binding one host plant DNA-base pair.

Scholze and Boch,2010;..

Structure of bacterial *avr* genes
avrBs3 family

- avrBs3 belongs to a gene family which has members in other species and pathovars *of Xanthomonas*.
- The protein products of these genes:

1. share 90-97 per cent sequence identity,
2. the differences occurring mainly within the 34-amino-acid repeats.

- All members of the avrBs3 family contain:

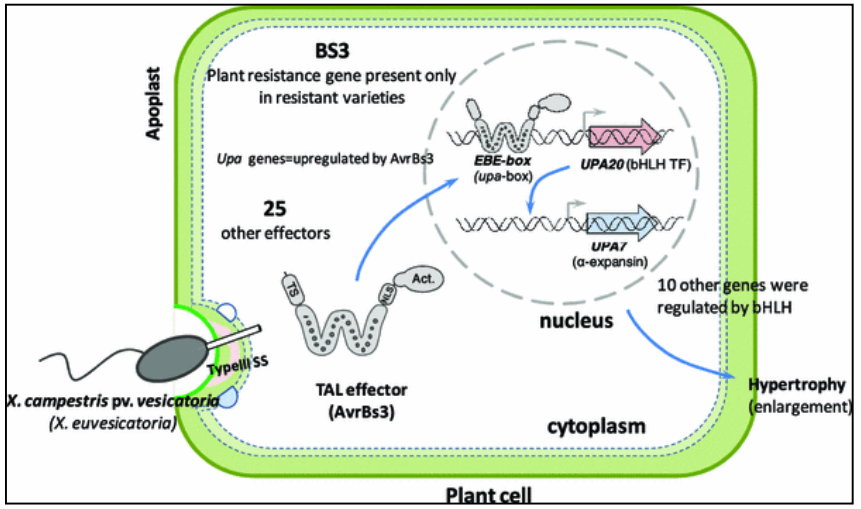
1. Nuclear localization signals (NLS),and
2. An acidic transcriptional activation domain (ACD), which are usually found only in eukaryotes.

- Mutations in either of domains of avrBs3 normally abolish recognition by plants carrying the cognate resistance gene.

AvrBs3 TAL effectors

Many plant-pathogenic xanthomonads rely on Transcription Activator-Like (TAL) effectors to colonize their host

- AvrBs3 TAL effector protein is secreted into the plant cell via a Type III secretion system.
- The internal natural nuclear localization signal of AvrBs3 leads to import to the nucleus, where this TALE searches for the base pair sequence recognized by the internal RVD (Repeat Variable Di-residue) in the DNA binding domain.
- Upon binding of the TAL effector to plant genes termed UPA (up-regulated by AvrBs3), transcription is initiated, leading to physiological effects in the infected plant cell such as hypertrophy.



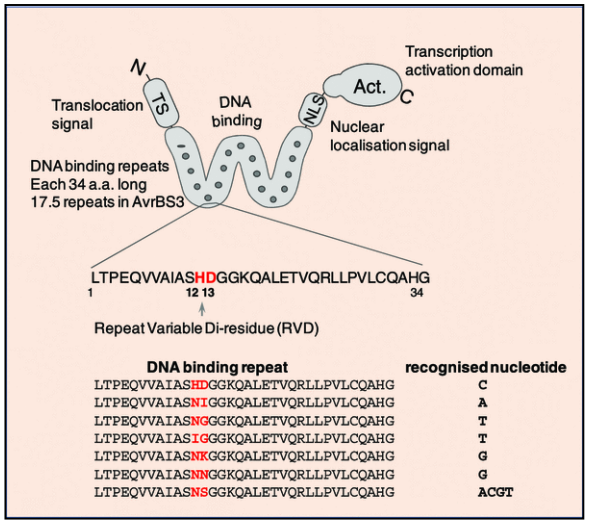
Transcription activator-like effectors (TALEs) are composed of modular functional domains, with each connected to the natural function of these proteins as virulence effectors.

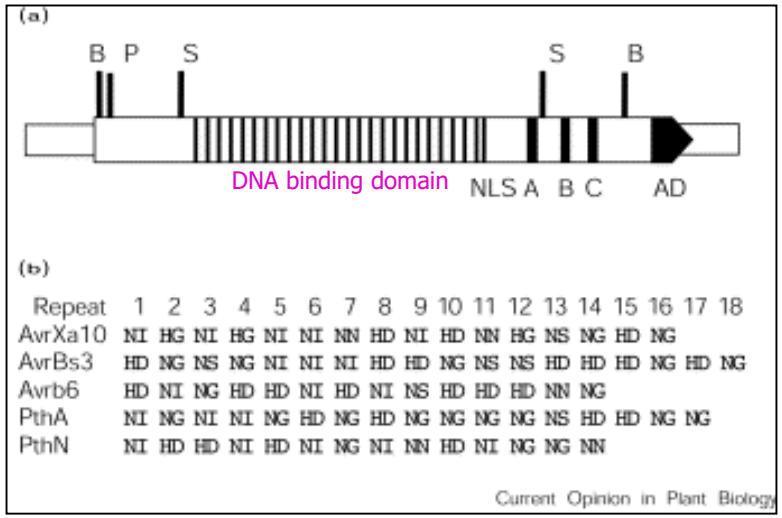
Fichtner *et al.*,2014

Structure of bacterial *avr* genes

Map of the phenotype gene in the *avrBs3* family

- AvrBs3 has a central domain that is highly repetitive, containing 17.5 nearly identical 34-amino-acid repeats in selected members of *avrBs3* family.
- Members of the TAL effector family differ differing in the 12th and 13th a.a. (Repeat Variable Di-residue, RVD) in the DNA binding domain.
- Functional nuclear localization signals (NLSs) are required for the avirulence activity of *avrBs3* (*X. vesicatoria*).
- AD=acidic transcriptional domain.



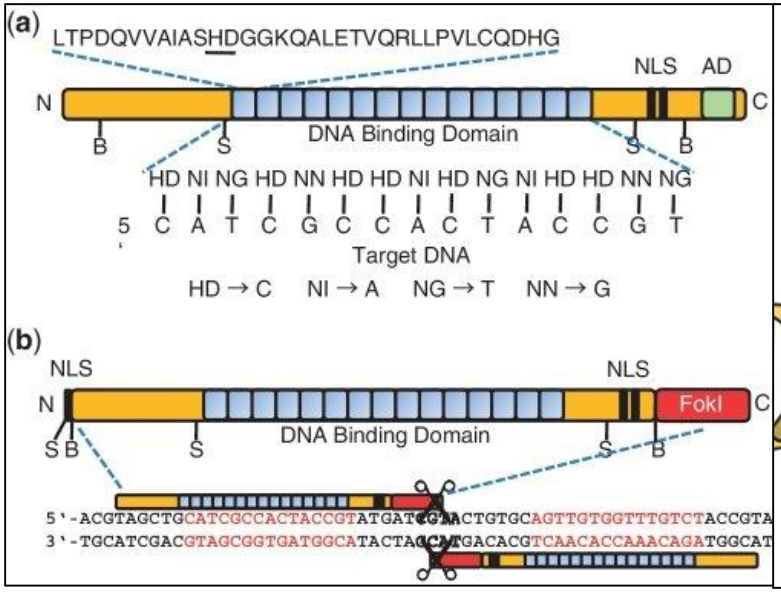


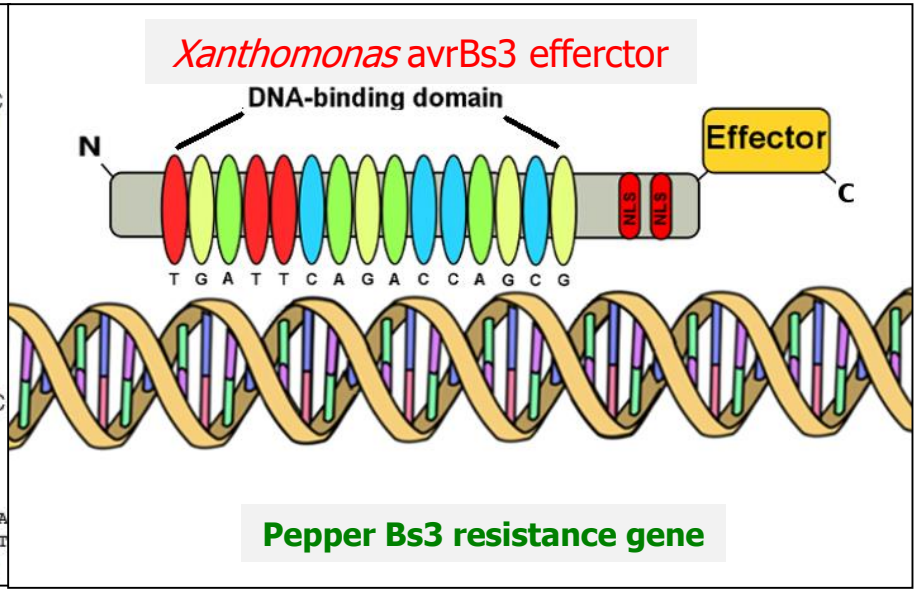
Structure of bacterial *avr* genes

Map of the phenotype gene in the *avrBs3* family

Domains of TAL effector AvrBs3

- One repeat from pathogen effector (underlined HD in figure a) recognizes one base pair on the host plant DNA (A, C, G, T) according to the repeat specificity and aligned.

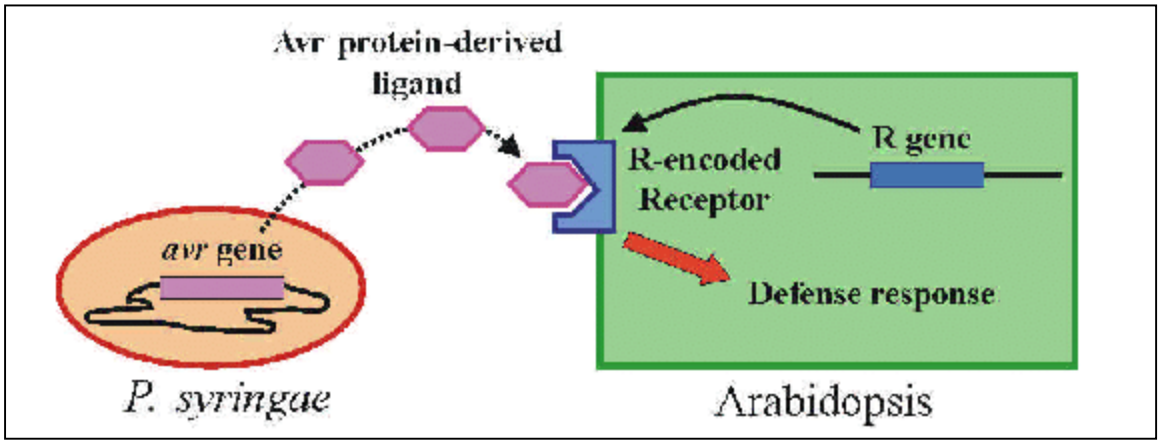




TAL effectors have several motifs normally associated with eukaryotes including multiple nuclear localization signals and an acidic activation domain.

The *avr* genes
Virulence or an avirulence factor

- The same *avr* gene may function as a virulence or an avirulence factor.
- If a particular *avr* effector in a normally virulent pathogen strain causes the pathogen to be recognized by a host R protein the effector is referred to as an avirulence protein.

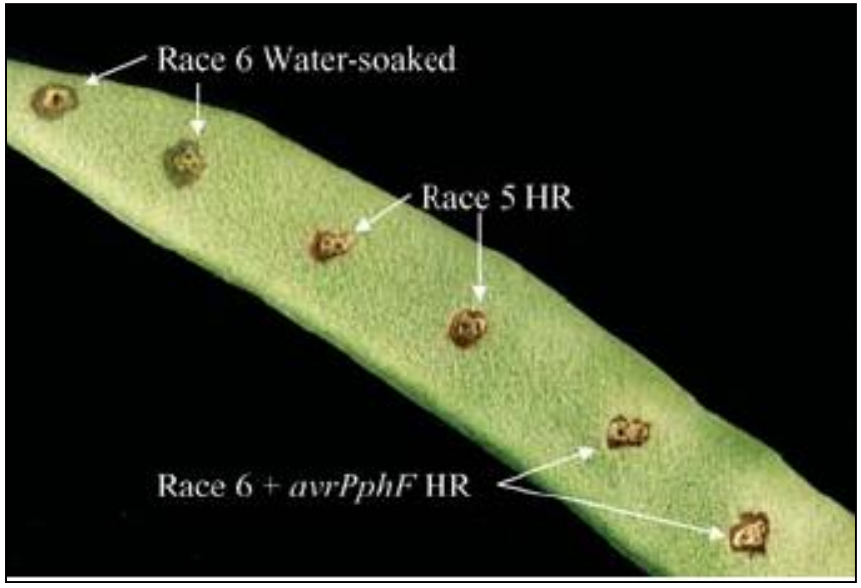


Katagiri *et al.*,2005

Cloning of the avirulence gene avrPphF from *P. phaseolicola* race 5

- Cloning of the avirulence gene avrPphF from *Pseudomonas phaseolicola* race 5.

1. Race 6 gives a susceptible water-soaked reaction, whereas
2. Race 5 gives a hypersensitive response (HR).
3. The HR was conferred on race 6 when transformed with a clone containing avrPphF (race 6+avrPphF).



Strange,2003

Plant genome informations

***R* genes/R proteins**

R gene-mediated resistance
Plants and animals

- The majority of disease resistance genes in plants encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins.
- The rapid evolution of plant immunity systems has led to enormous gene diversification.

Jennifer *et al.*,2007; Sanseverino *et al.*,2010;..

R gene-mediated resistance
Plants and animals

- Resistance genes (R-genes) act as an immune system in plants by recognizing pathogens and inducing defensive pathways.
- R-genes originate from a phylogenetically ancient form of immunity that is common to plants and animals.
- The largest class encodes nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins that are structurally related to proteins involved in innate immunity in animals.

Jennifer *et al.*,2007; Sanseverino *et al.*,2010;..

R genes in plant genome

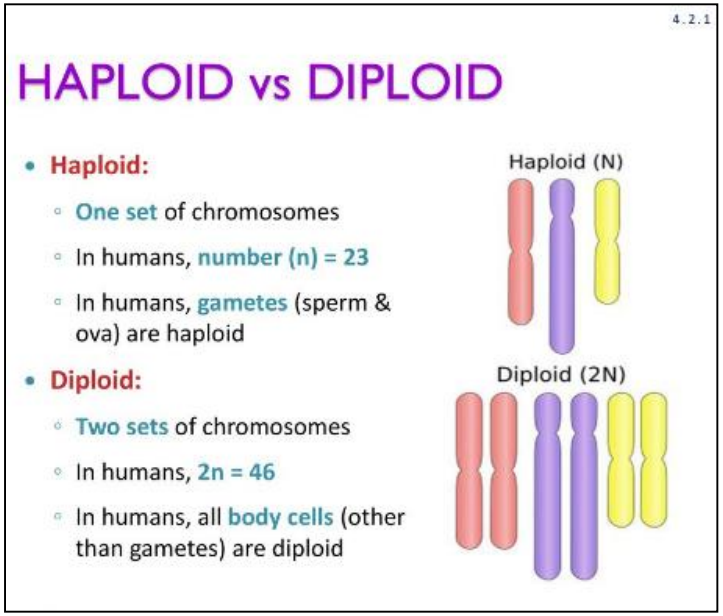
Identification of plant genes that recognize essential virulence components of pathogens

- In theory, durable resistance would be expected to be a consequence of recognition of some component of the pathogen that is essential for its virulence.

Genes vs. allele

Different version of genes are called allele

- Diploid organisms typically have two alleles for a trait.
- Diploid is a cell or organism that has paired chromosomes, one from each parent.
- In humans, cells other than human sex cells, are diploid and have 23 pairs of chromosomes.
- An allele is an alternative form of a gene (one member of a pair) that is located at a specific position on a specific chromosome.

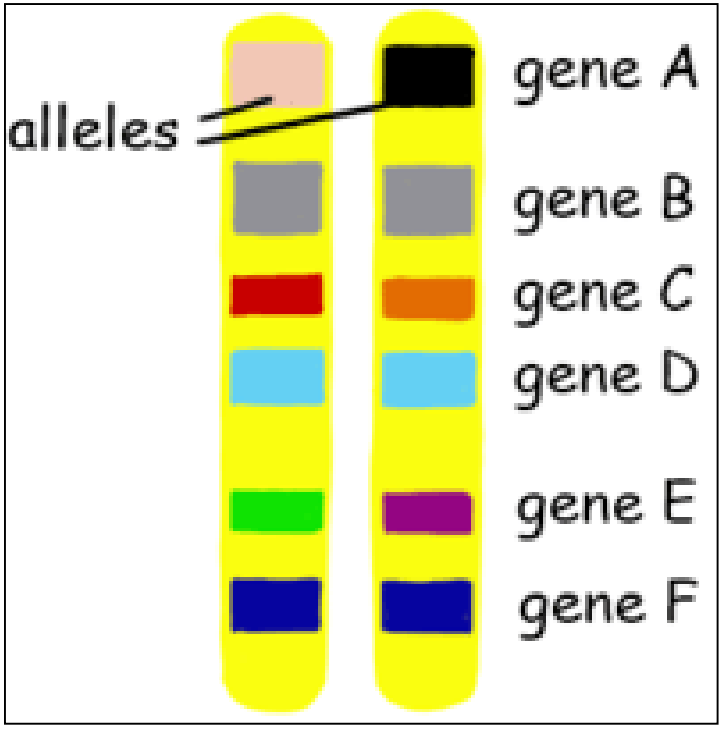


Genes vs. allele

Different version of genes are called allele

- Diploid organisms typically have two alleles for a trait.
- These DNA coding's determine distinct traits that can be passed on from parents to offspring through sexual reproduction.

1. For some genes, nearly everyone has the same allele.
2. For other genes, alleles vary; there might be two, three, or more alleles.



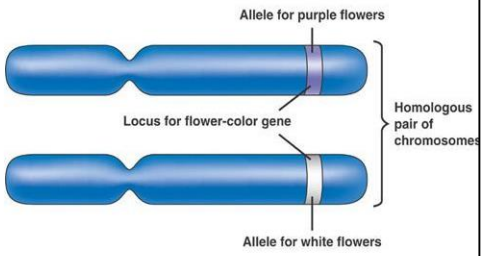
Evolution is a Myth;Quora

Genes vs. allele

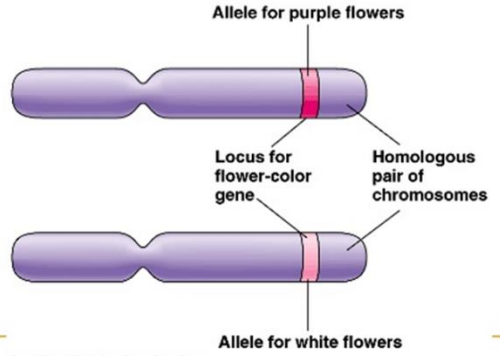
Different version of genes are called allele

Genes and Alleles

- Gene: a heritable factor that controls a specific characteristic
  - Located in specific places on chromosomes
  - Humans have roughly 20,000-25,000 genes.
- Alleles: alternative forms of gene
- Example:
  - Gene: eye color
  - Alleles: blue vs. brown



- Different versions of genes are called **alleles**



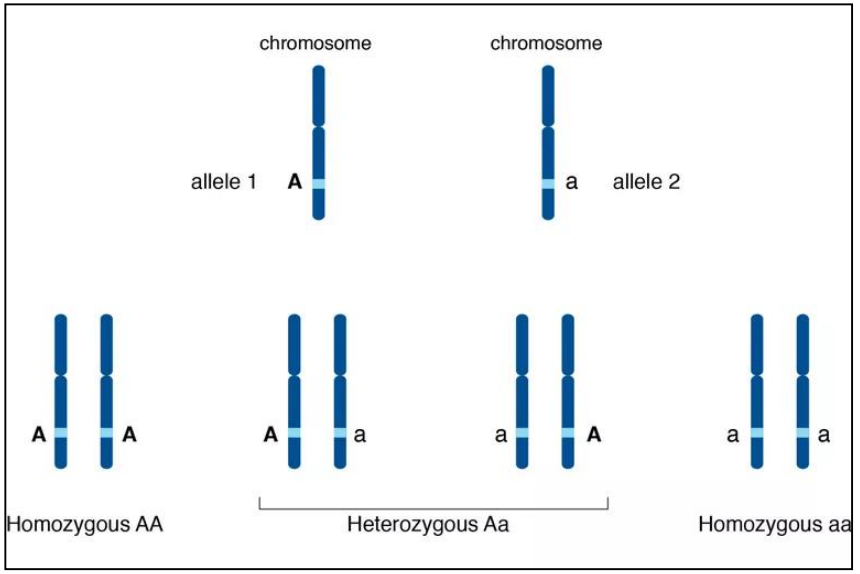
Evolution is a Myth;..

Genes vs. allele

Dominant and Recessive Alleles

Homozygous and heterozygous

- When allele pairs are the same, they are homozygous.
- When the alleles of a pair are heterozygous, the phenotype of one trait may be dominant and the other recessive.
- The dominant allele is expressed and the recessive allele is masked. This is known as complete genetic dominance.
- In heterozygous relationships where neither allele is dominant but both are completely expressed, the alleles are considered to be co-dominant.

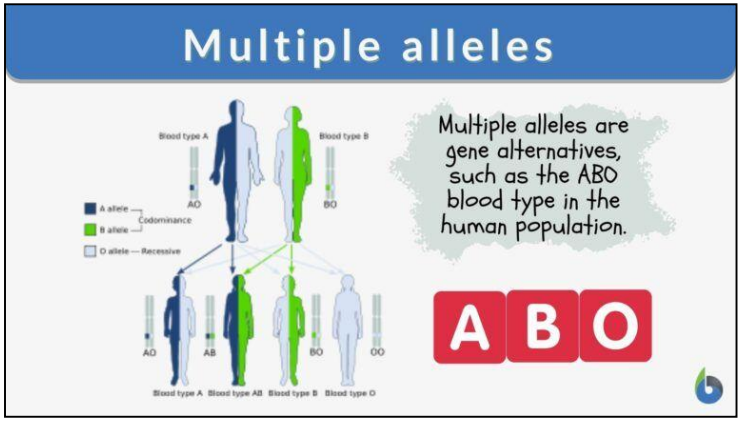


Thought Co.

Genes vs. allele

Multiple alleles in human blood group

- While most genes exist in two allele forms, some have multiple alleles for a trait.
- A common example of this in humans is ABO blood type.
- ABO blood types exist as three alleles, which are represented as (IA, IB, IO).
- These multiple alleles are passed from parent to offspring such that one allele is inherited from each parent.
- There are four phenotypes (A, B, AB, or O) and six possible genotypes for human ABO blood groups.

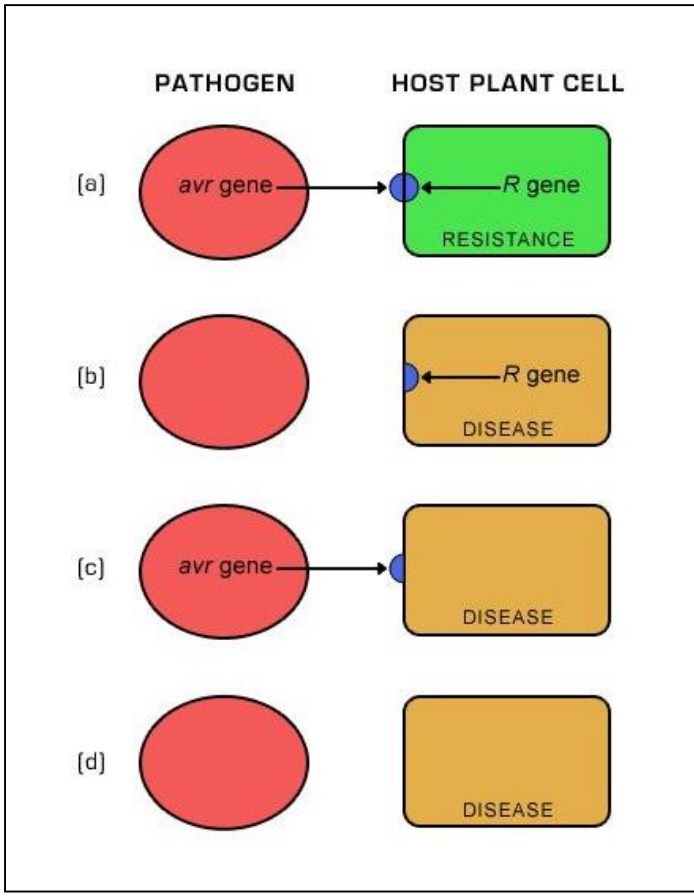


Allele from Parent 1	Allele from Parent 2	Genotype of offspring	Blood types of offspring (Phenotype)
A	A	AA	A
A	O	AO	A
B	B	BB	B
B	O	BO	B
A	B	AB	AB
O	O	OO	O

Gene-for-Gene theory

R gene and Avr gene

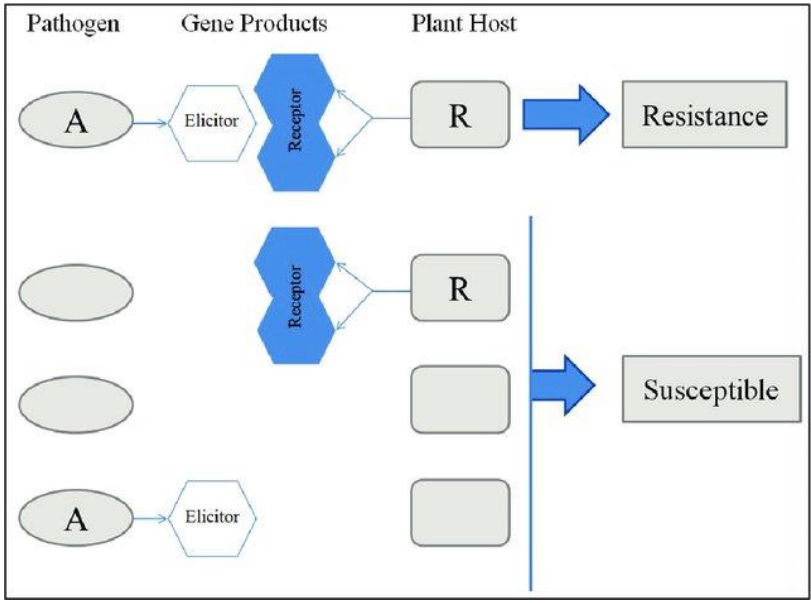
A. Resistance occurs if there is R gene present in host plants.
B. Disease occurs if there is no gene-for-gene recognition because the pathogen has no Avr allele matching an R allele of the plant.
C. Disease occurs if the plant R alleles do not match the Avr alleles on the pathogen,
D. Disease occurs if neither have recognition alleles.



Andrew Barrett

Molecular Model of the Gene for Gene Interaction Modified from Staskawicz *et al.*,1995

- Resistance occurs only if specific recognition occurs between host (R) and pathogen (A) gene products.
- Disease occurs if there is no gene-for-gene recognition because the pathogen has no Avr allele matching an R allele of the plant.
- Disease occurs if the plant R alleles do not match the Avr alleles on the pathogen,
- Disease occurs if neither have recognition alleles.

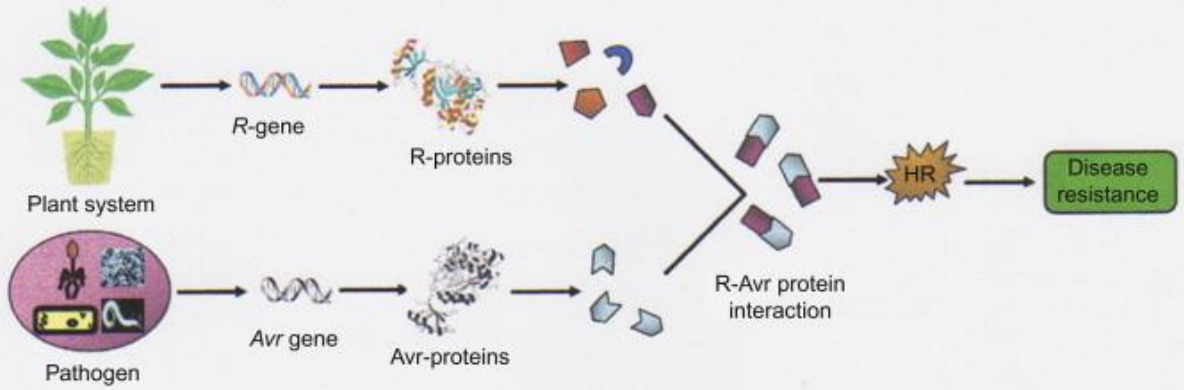


Any alteration in gene products leads to the susceptible phenotype.

Ijaz *et al.*,2018

Quadratic Check Showing Interaction Types in Gene for Gene Reaction Modified from Keller et al.,2000

(A)



(B)

Host phenotype	Pathogen phenotype: Avirulent AA	Avirulent Aa	Virulent Aa
Susceptible Rr	+	+	+
Resistant Rr	–	–	+
Resistant RR	–	–	+

A. For specific resistance(+) to occur, complementary pairs of dominant genes, one in the host and the other in the pathogen, are required.

B. A loss or alteration in the host resistance (R) gene or in the pathogen avirulence (Avr) gene leads to disease or compatibility (-).

Flor gene-for-gene hypothesis
Quadratic check

Pathogen genotype	Host genotype	
	RR or *Rr*	*rr*
avr gene present	–	+
avr gene absent*	+	+

R/*r*: resistance; *avr*: avirulence; +: compatible (disease); –: incompatible (resistant).
* Would also include mutations in *avr* genes, which can be regarded as inactive alleles, resulting in compatibility in gene-for gene combinations.

Vivian and Arnold,2000

Flor gene-for-gene hypothesis
Quadratic check

Gene-for-gene hypothesis Quadratic Check

Pathogen Avirulence (virulence) genes	Plant genes Resistance (susceptibility)	
	R (Resistant)	r (susceptible)
A (Avirulent)	AR (-)	Ar(+)
a (virulent)	aR(+)	ar(+)

where, – = Resistance
+ = Susceptible

(Agrios 2007)

Gene-for-gene hypothesis Multifactor Interactions

Avirulence/virulence	Resistance/susceptibility			
	R1 R2	r1 R2	R1r2	r1r2
A1A2	-	-	-	+
a1A2	-	-	+	+
A1a2	-	+	-	+
a1a2	+	+	+	+

where, – = Resistance
+ = Susceptible

(Agrios 2007)

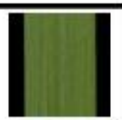
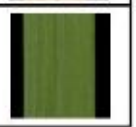
Possible combinations

AA – homozygous dominant
Aa, aA – heterozygous
aa – homozygous recessive

RACE - SPECIFIC GENETIC INTERACTIONS

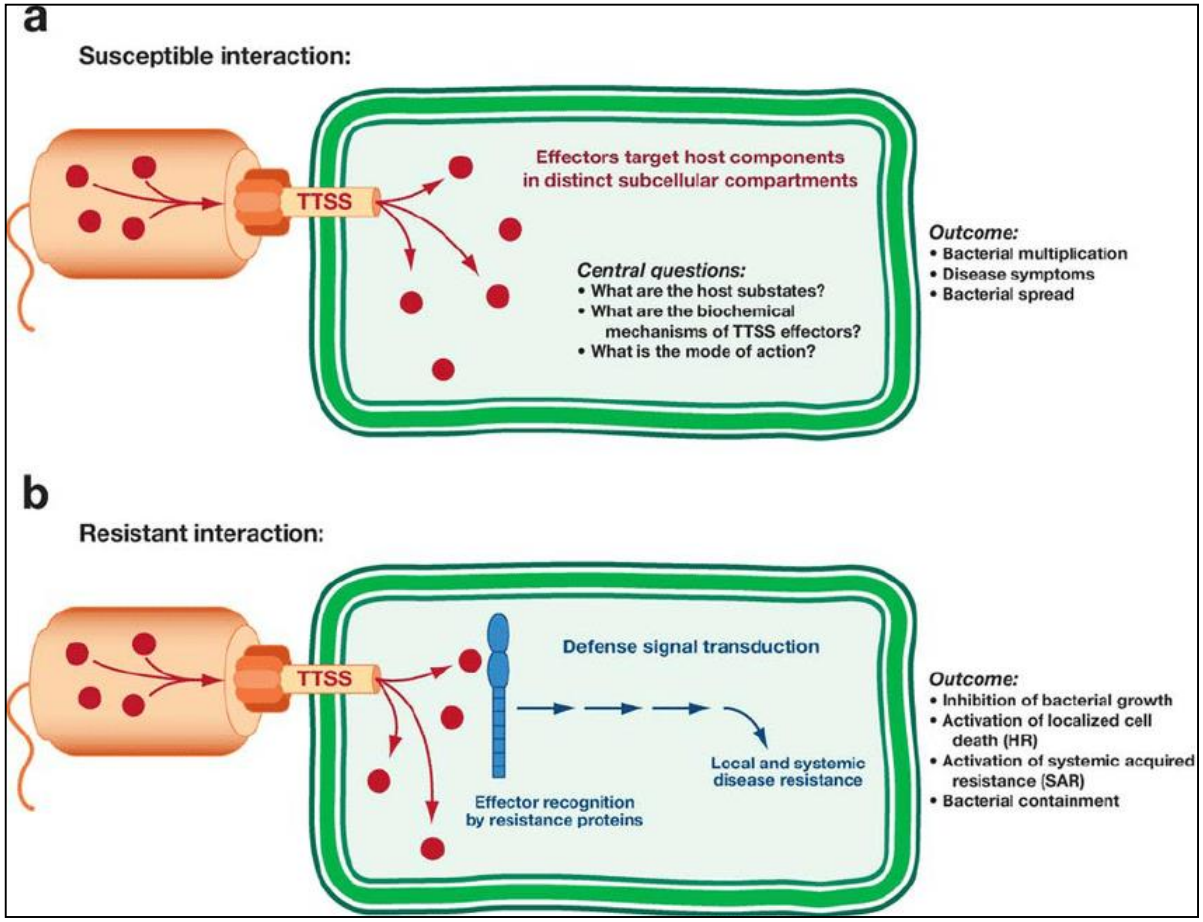
FLOR's 'Gene-for-Gene' Hypothesis

Host plant genotype

Pathogen genotype	*R1 r2*	*r1 R2*
Avr1, avr2	I 	C 
avr1, Avr2	C 	I 

I - incompatible - no disease
C - compatible - disease

Gene-for-gene hypothesis
Type III secretion system (TTSS)



Mudgett,2005

R genes in plant genome

- Resistance gene are often present as gene clusters of different specificities in the plant genome.
- Majority of these *R* genes show conserved DNA sequences and amino acid domains irrespective of whether they confer resistance to bacterial, fungal, viral, or nematode pathogens.

Plant disease resistance
R genes

- Plants have many, highly variable resistance (*R*) gene loci, which provide resistance to a variety of pathogens.
- The first *R* gene to be cloned, maize (*Zea mays*) *Hm1*, was published over 25 years ago, and since then, many different *R* genes have been identified and isolated.
- Over 60 plant disease resistance (*R*) genes have been cloned from different monocot and dicot plant species.

Gene-for-gene resistance

Resistance (*R*) genes

NOD-like receptors (NLRs) or NBD-LRR domain-containing

- More than 100 *R* genes have been cloned from various species, and most of them contain:

1. a conserved nucleotide-binding domain (NB-ARC), and
2. leucine-rich repeat domain (LRR).

NB-ARC: a novel signaling motif shared by plant resistance gene products and regulators of cell death in animals.

Borrelli *et al.*,2018

R genes identified
Against *X. axonopodis* pv.*malvacearum*

- Strains of *Xanthomonas axonopodis* pv. *malvacearum* exhibit gene-for-gene (also known as cultivar or race) specificity.
- There are at least 19 well-described races of the pathogen.
- At least 16 different resistance (*R*) genes identified.
- Different *Xanthomonas axonopodis* pv. *malvacearum* races contain different combinations of avirulence (*avr*) genes that:

1. Define the race, and
2. Determine cultivar specificity.

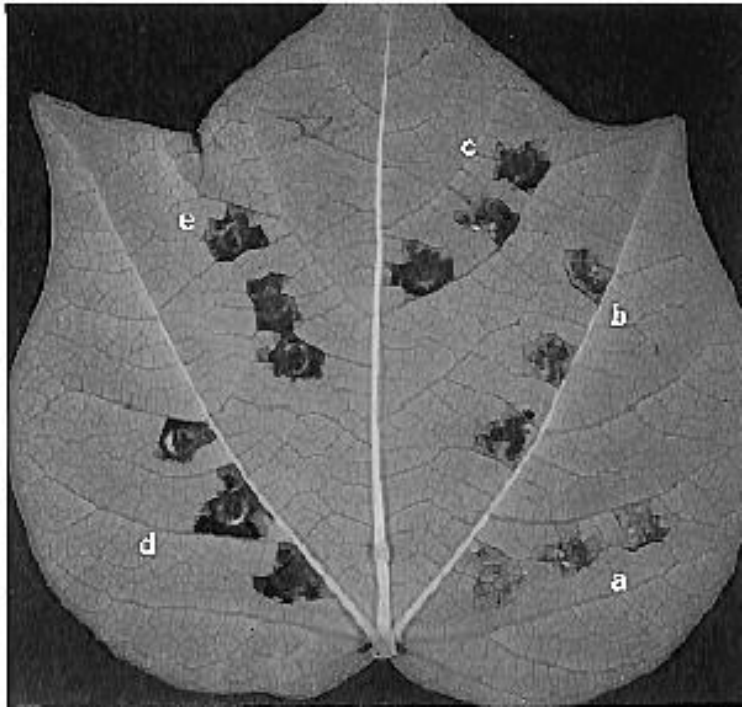
R genes identified

Against *X. axonopodis* pv.*malvacearum*

- 10 *avr* genes were cloned and characterized from a single North American strain of *X. axonopodis* pv. *malvacearum*, XcmH.
- Most of these *avr* genes genetically "recognize" multiple *R* gene loci in an *avr* gene-for-*R* genes (plural *R* genes) manner.
- These ten *avr* genes belong to a large family of *Xanthomonas avr/pth* (avirulence (*avr*)/pathogenicity (*pth*) gene family.

X. a. pv. *malvacearum*
pthN and *pthN2*

- 10 genes belong to a large family of *Xanthomonas avr/pth* (avirulence/pathogenicity genes) were found in *X. a.* pv. *malvacearum*.
- Water-soaking symptoms caused by *pthN* and *pthN2*.
- Photo taken 5 days after inoculation.



Plant disease resistance genes

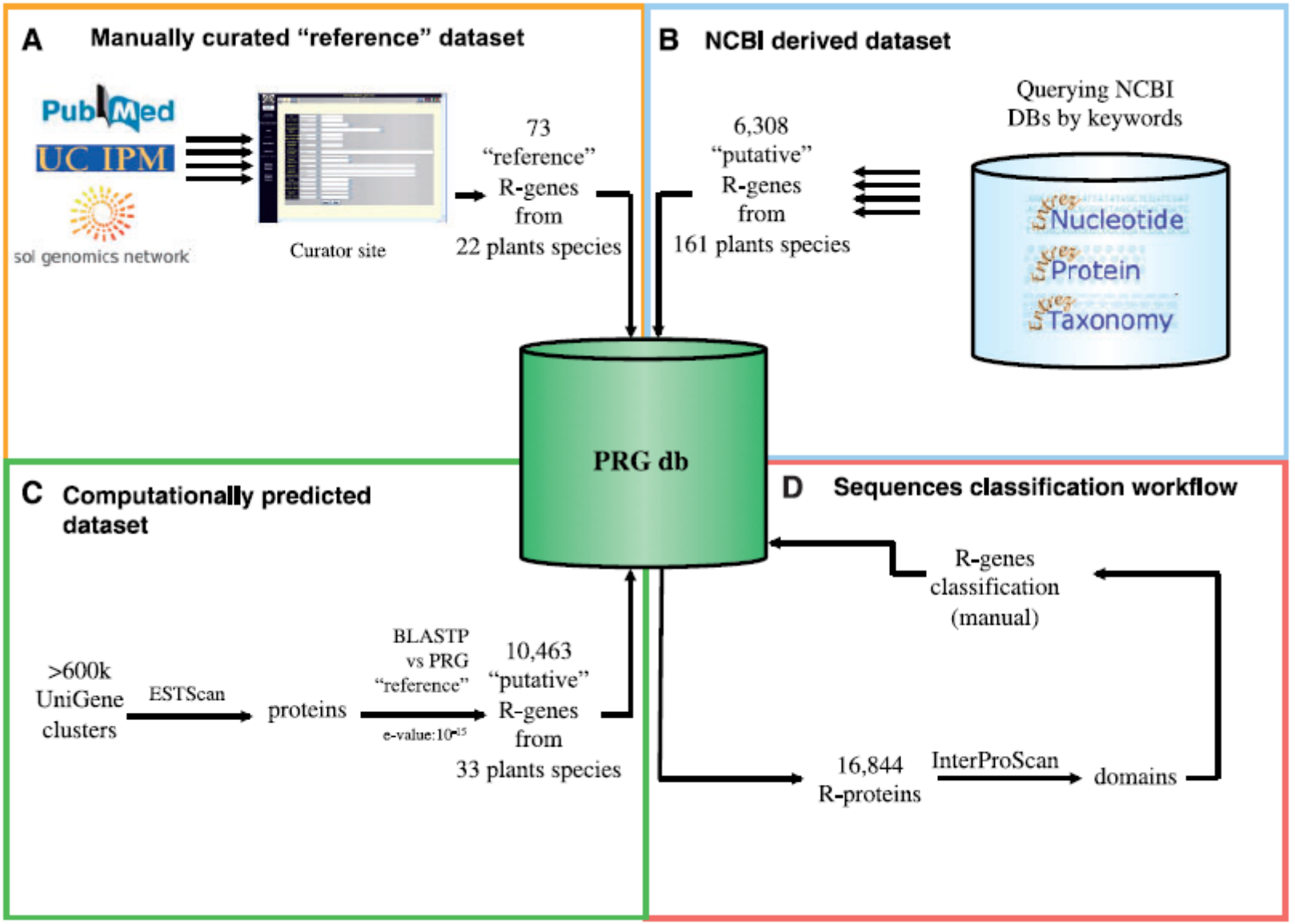
Plant resistance gene database (PRGdb)

- The plant resistance gene database (PRGdb), is the first comprehensive bioinformatics resource dedicated to known and predicted plant disease resistance genes.
- It is a web accessible open-source (http://www.prgdb.org) database providing a comprehensive overview of resistance genes (R-genes) in plants.
- The complete database includes:
- A set of 73 manually curated reference R-genes,
- 6308 putative R-genes collected from NCBI, and
- 10463 computationally predicted putative R-genes.

Sanseverino *et al.*,2010

Plant disease resistance genes

Plant resistance gene database (PRGdb)



Sanseverino *et al.*,2010

Plant resistance gene database (PRGdb)

A schematic view of the PRG database showing the origin of dataset used and the sequences characterization

A. The manually curated dataset that contains 73 literature cited R-genes from 22 different plants.
B. The NCBI dataset containing 6308 sequences related to reference R-genes retrieved by the NCBI database.
C. The computationally predicted dataset using the DRAGO pipeline containing 10 463 putative R-genes.
D. Workflow of conserved domain analysis and sequence classification.

Sanseverino *et al.*,2010

R gene-mediated resistance
Defect

- Unfortunately, *R* genes are often quickly defeated by co-evolving pathogens.
- Many *R* genes **recognize only** a limited number of pathogen strains and therefore do not provide broad-spectrum resistance.
- Introgression of *R* genes into elite cultivars by conventional breeding is a lengthy process.

R gene-mediated resistance
Prospect

- However, recent molecular-level insights into the function of R proteins and downstream signal transduction pathways might provide strategies to remedy these deficiencies.

Plant disease resistance

R genes

R genes functions

- Roles of *R* genes are presumed to:
- Most products of resistance genes against bacteria are predicted to reside in the cytoplasm.
- The R protein and its matching *avr* gene product often co-localise.
- Upon pathogen recognition, many defense responses, such as the HR, PR protein accumulation and an oxidative burst are activated.

Plant disease resistance

R genes

R genes functions

- Roles of *R* genes are presumed to:

1. Effector recognition- enable plants to detect *avr*-gene-specified pathogen molecules (i.e. gene-for-gene resistance).
2. Initiate signal transduction to activate defenses.
3. Have the capacity to evolve new *R* gene specificities rapidly.

Plant disease resistance

R genes

R genes functions

- Roles of *R* genes are presumed to:

1. Most R proteins recognize a specific effector, but
2. Some function in pairs that recognize multiple effectors.

- *Arabidopsis thaliana* TIR-NB-LRR proteins RRS1-R and RPS4 together recognize two bacterial effectors:

1. AvrRps4 from *Pseudomonas syringae*, and
2. PopP2 from *Ralstonia solanacearum*.

Saucet *et al.*,2015

Plant functional resistance genes identified to date in the plant kingdom with indication of donor species, related disease and pathogen

Gene Name	Donor Species	Disease	Pathogen
Asc1	*Solanum lycopersicum*	Alternaria stem canker	*Alternaria alternata*
At1	*Cucumis melo*	Cucurbit downy mildew	*Pseudoperonospora cubensis*
At2	*Cucumis melo*	Cucurbit downy mildew	*Pseudoperonospora cubensis*
Bs2	*Capsicum chacoense*	Bacterial spot	*Xanthomonas campestris pv. vesicatoria str. 85-10*
Bs3	*Capsicum annuum*	Bacterial spot	*Xanthomonas campestris pv. vesicatoria str. 85-10*
Bs3-E	*Capsicum annuum*	Bacterial spot	*Xanthomonas campestris pv. vesicatoria str. 85-10*
Bs4	*Solanum lycopersicum*	Bacterial spot	*Xanthomonas campestris*
Cf2	*Solanum pimpinellifolium*	Leaf mould	*Passalora fulva*
Cf4	*Solanum habrochaites*	Leaf mould	*Passalora fulva*
Cf4A	*Solanum habrochaites*	Leaf mould	*Passalora fulva*
Cf5	*Solanum lycopersicum var. cerasiforme*	Leaf mould	*Passalora fulva*
Cf9	*Solanum pimpinellifolium*	Leaf mould	*Passalora fulva*
Cf9B	*Solanum pimpinellifolium*	Leaf mould	*Passalora fulva*
Dm-3	*Lactica sativa*	Downy mildew	*Bremia lactucae*
EFR	*Arabidopsis thaliana*	Eliciting bacteria	*Bacteria with flagellum*
ER-Erecta	*Arabidopsis thaliana*	Bacterial wilt (Arabidopsis)	*Ralstonia solanacearum*
FLS2	*Arabidopsis thaliana*	Eliciting bacteria	*Bacteria with flagellum*
Gpa2	*Solanum tuberosum*	Yellow potato cyst nematode	*Globodera*
Gro1.4	*Solanum tuberosum*	Late blight potato	*Phytophthora infestans*
Hero	*Solanum lycopersicum*	Yellow potato cyst nematode	*Globodera*

Sanseverino *et al.*,2010

Plant functional resistance genes identified to date in the plant kingdom with indication of donor species, related disease and pathogen

Hm1	*Zea mays*	Leaf spot	*Bipolaris zeicola*
Hm2	*Zea mays*	Leaf spot	*Bipolaris zeicola*
HRT	*Arabidopsis thaliana*	Turnip crinkle virus	*Turnip crinkle virus*
Hs1	*Beta procumbens*	Beet cyst nematode	*Heterodera schachtii*
I2	*Solanum lycopersicum*	Fusarium wilt	*Fusarium oxysporum*
L6	*Linum usitatissimum*	Flax rust	*Melampsora lini*
LeEIX1	*Solanum lycopersicum*	Eliciting fungus	*Fungal ethylene-inducing xylanase*
LeEIX2	*Solanum lycopersicum*	Eliciting fungus	*Fungal ethylene-inducing xylanase*
M	*Linum usitatissimum*	Flax rust	*Melampsora lini*
Mi1.2	*Solanum lycopersicum*	Root-knot nematode	*Meloidogyne, Paratrichodorus minor*
MLA10	*Hordeum vulgare*	Powdery mildew (barley)	*Blumeria graminis*
Mlo	*Hordeum vulgare*	Powdery mildew (barley)	*Blumeria graminis*
N	*Nicotiana glutinosa*	Tobacco mosaic Virus	*Tobacco mosaic virus*
P2	*Linum usitatissimum*	Flax rust	*Melampsora lini*
PEPR1	*Arabidopsis thaliana*	Damping off	*Pythium*
PGIP	*Phaseolus vulgaris*	Eliciting fungus	*Fungus producing polygalacturonases*
Pi33	*Oryza sativa*	Rice blast disease	*Magnaporthe grisea*
Pi-ta	*Oryza sativa Japonica Group*	Rice blast disease	*Magnaporthe grisea*
Prf	*Solanum pimpinellifolium*	Bacterial speck	*Pseudomonas syringae*
Pto	*Solanum pimpinellifolium*	Bacterial speck	*Pseudomonas syringae*

Sanseverino *et al.*,2010

Plant functional resistance genes identified to date in the plant kingdom with indication of donor species, related disease and pathogen

R1	*Solanum demissum*	Late blight tomato	*Phytophthora infestans*
R3a	*Solanum tuberosum*	Late blight tomato	*Phytophthora infestans*
RCY1	*Arabidopsis thaliana*	Cucumber mosaic virus	*Cucumber mosaic virus*
RFO1	*Arabidopsis thaliana*	Fusarium wilt	*Fusarium oxysporum*
Rmd-c	*Glycine max*	Powdery mildew	*Microsphaera sparsa*
RPG1	*Hordeum vulgare*	Stem rust	*Puccinia Graminis*
Rpi-blb1	*Solanum bulbocastanum*	Late blight tomato	*Phytophthora infestans*
Rpi-blb2	*Solanum bulbocastanum*	Late blight tomato	*Phytophthora infestans*
RPM1	*Arabidopsis thaliana*	Bacterial blight	*Pseudomonas syringae*
RPP13nd	*Arabidopsis thaliana*	Downy mildew	*Hyaloperonospora parasitica*
RPP4	*Arabidopsis thaliana*	Downy mildew	*Peronospora parasitica*
RPP5	*Arabidopsis thaliana*	Downy mildew	*Hyaloperonospora parasitica*
RPP8	*Arabidopsis thaliana*	Downy mildew	*Hyaloperonospora parasitica*
Rps1-k-1	*Glycine max*	Phytophthora root	*Phytophthora sojae*
Rps1-k-2	*Glycine max*	Phytophthora root	*Phytophthora sojae*
Rps2	*Arabidopsis thaliana*	Bacterial blight	*Pseudomonas syringae*
Rps4	*Arabidopsis thaliana*	Bacterial blight	*Pseudomonas syringae*
RPS5	*Arabidopsis thaliana*	Bacterial blight	*Pseudomonas syringae*
RPW8.1	*Arabidopsis thaliana*	Powdery mildew	*Golovinomyces cichoracearum*
RPW8.2	*Arabidopsis thaliana*	Powdery mildew	*Golovinomyces cichoracearum*
RRS1	*Arabidopsis thaliana*	Bacterial wilt	*Ralstonia solanacearum*

Sanseverino *et al.*,2010

Plant functional resistance genes identified to date in the plant kingdom with indication of donor species, related disease and pathogen

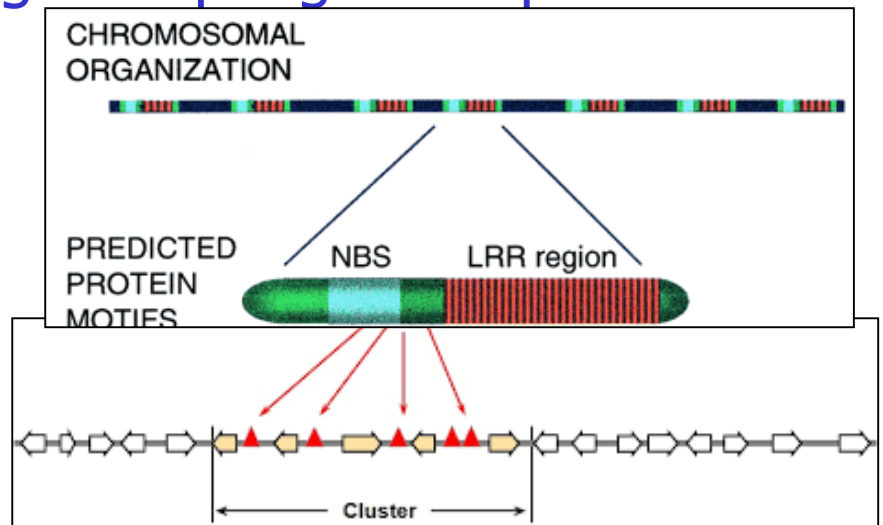
RTM1	*Arabidopsis thaliana*	Synergistic disease syndromes	*Tobacco etch virus*
RTM2	*Arabidopsis thaliana*	Synergistic disease syndromes	*Tobacco etch virus*
Rx	*Solanum tuberosum*	Latent mosaic	*Potato virus X*
Rx2	*Solanum acaule*	Latent mosaic	*Potato virus X*
RY1	*Solanum tuberosum subsp andigena*	Potato virus Y	*Potato virus Y*
Sw5	*Solanum lycopersicum*	Tomato spotted wilt	*Tomato spotted wilt virus*
Tm2	*Solanum lycopersicum*	Tobacco mosaic virus	*Tobacco mosaic virus*
Tm2a	*Solanum lycopersicum*	Tobacco mosaic virus	*Tobacco mosaic virus*
Ve1	*Solanum lycopersicum*	Verticillium wilt potato	*Verticillium*
Ve2	*Solanum lycopersicum*	Verticillium wilt potato	*Verticillium*
Xa1	*Oryza sativa*	Bacterial blight	*Xanthomonas oryzae*
Xa21	*Oryza sativa Indica group*	Bacterial blight	*Xanthomonas oryzae*

Sanseverino *et al.*,2010

R gene-mediated resistance

R gene/proteins structure

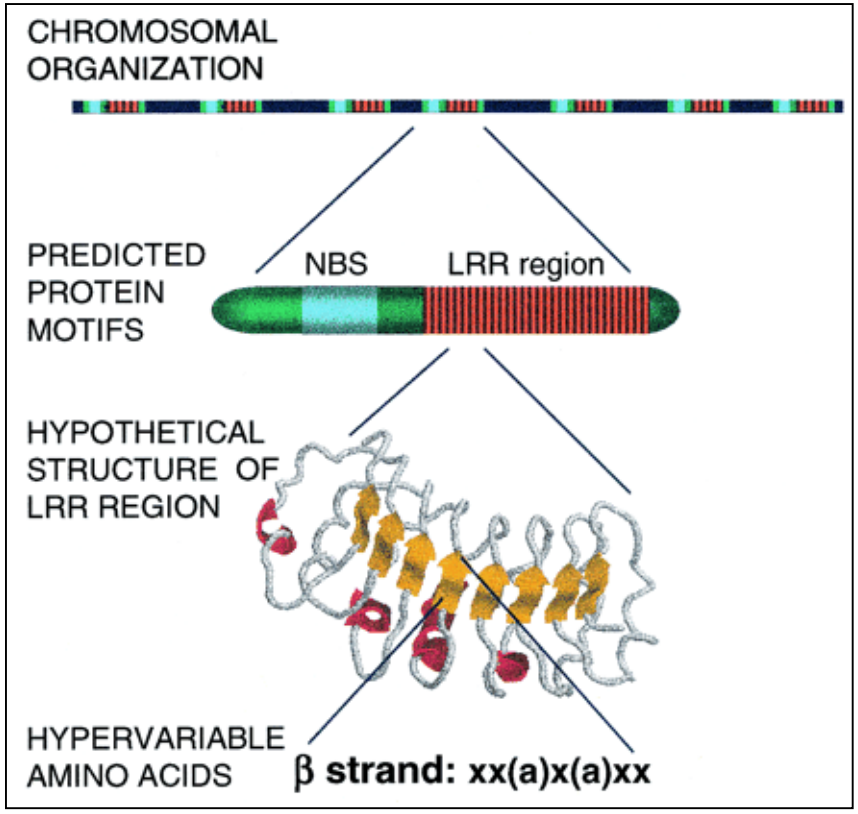
- Plants have evolved various mechanisms that protect them from pathogen invasion and colonization.
- R genes encode receptors containing a nucleotide-binding site and leucine-rich repeats (NBS-LRR).
- Most R genes are organized into tight clusters containing multiple gene copies.



The organization and structure of resistance genes and their products

***R*-gene products are postulated to have receptor and effector domains**

- Leucine-rich repeats (LRR) consist of 2-45 motifs of 20-30 amino acids in length that generally folds into an arc or horseshoe shape.
- This hypothetical structure shows the thyrotropin receptor.
- In the consensus sequence for the β-strand, (x) any amino acid; (a) aliphatic residues.



Thyrotropin: A tripeptide hormone. Aliphatic: Acyclic or cyclic, not aromatic carbon compounds.

Michelmore and Meyers,1998;..

R gene-mediated resistance

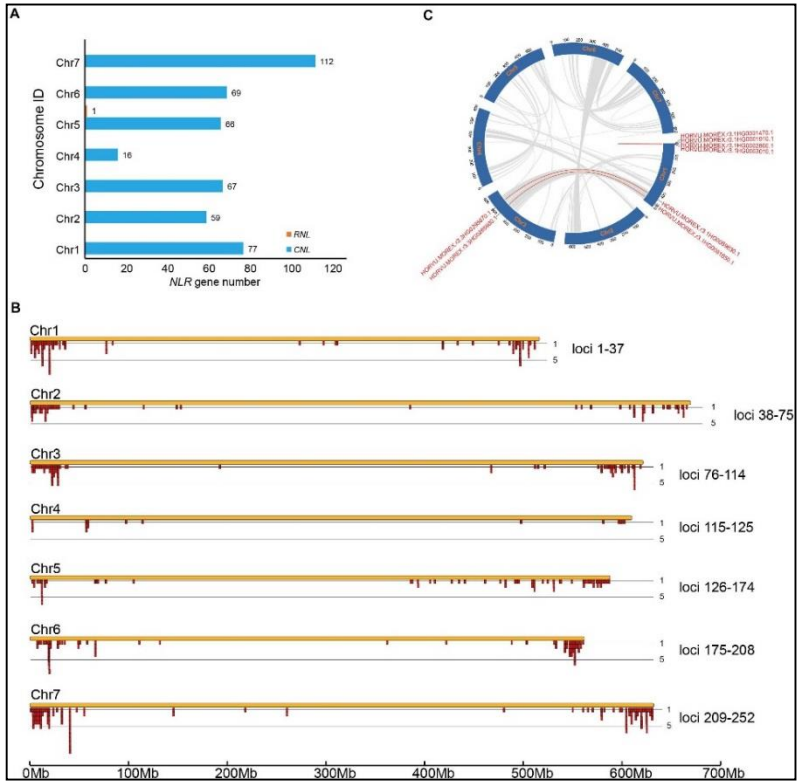
Chromosomal distribution of barley NB-LRR (NLRs) of R proteins

- NLR gene number variation among barley genomes.

A. Physical locations of *NLR* genes on barley chromosomes.

B. *NLR* genes within an interval less than 250 kb were treated as a cluster.

C. Syntenic relationship of the eight segmental-duplicated *NLR* genes.



NLRs were originally referred to as "Nod-like receptors," but we do not favor this name because it arose only in the mammalian literature.The official consensus nomenclature is that NLR stands for "NBD-LRR domain-containing".

Li *et al.*,2021

The organization and structure of resistance genes

Major classes (families) of R proteins

MAJOR CLASSES OF R PROTEINS

S. NO	MAJOR R-GENE CLASSES	EXAMPLE
1	NBS-LRR-TIR	N, L6, RPP5
2	NBS-LRR-CC	I2, RPS2, RPM1
3	LRR-TrD	Cf-9, Cf-4, Cf-2
4	LRR-TrD-Kinase	Xa21
5	TrD-CC	RPW8
6	TIR-NBS-LRR-NLS- WRKY	RRS1R
7	LRR-TrD-PEST-ECS	Ve1, Ve2
8	Enzymatic R-genes	Pto, Rpg1

LRR - Leucine rich repeats; **NBS** - Nucleotide-binding site; **TIR** -Toll/Interleukin-1- receptors; **CC** - Coiled coil; **TrD** -Transmembrane domain; **PEST** -Amino acid domain; **ECS** - Endocytosis cell signaling domain; **NLS** - Nuclear localization signal; **WRKY** -Amino acid domain; **HC toxin reductase** - Helminthosporium carbonum toxin reductase enzyme.

Gururani *et.al.*,2012

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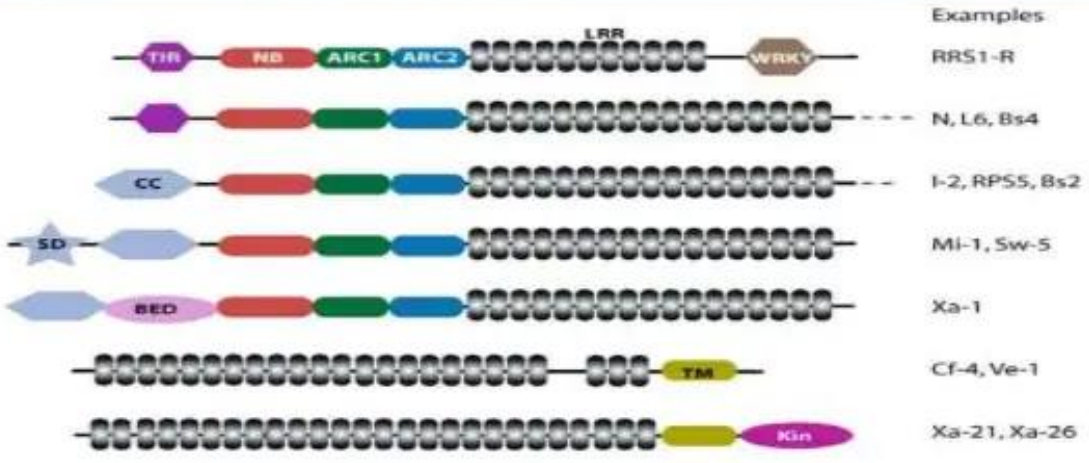
The eight major classes of resistance gene-encoding R-proteins and their domain arrangements

Plant disease resistance (*R*) genes encode proteins containing domains. e.g. TIR, NBS, and LRR

Domain	Function	Gene
LRR	Protein-protein interaction. Its major determinants of recognition specificity	*Pi-ta, Cf 4,9,5*
NBS	To bind ATP r GTP. Race specificity functions of R gene	*L6*
TIR	Race specificity functions of R gene	*L6*
CC	Involved in recognition of avr gene product	*RPW 8*



Schematic representation of domains found in plant LRR R proteins. Domains are not drawn to scale. TIR Toll/interleukin-1 receptor, CC coiled coil, NB nucleotide binding, ARC1/2 APAF1, R protein and CED4, LRR leucine rich repeat, SD solanaceous domain, BED BEAF/DREAF zinc finger domain, TM transmembrane, Kin kinase, WRKY WRKY transcription factor

The NB/NBS domain, also known as NB-ARC where ARC stands for APAF1 (apoptotic protease-activating factor-1)

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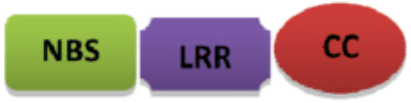
(Wladimir *et al.*, 2008)

The eight major classes of resistance gene-encoding R-proteins and their domain arrangements

Plant disease resistance (*R*) genes encode proteins containing domains. e.g. TIR, NBS, and LRR

Class	Arrangement of functional domains	Examples	
		Host	R-gene
CNL		Tomato	*I2, Mi-1.2*
TNL		Tobacco, *Arabidopsis*	*N gene, RPP5*
eLRR-TrD		Tomato	*Cf-9, Cf-4, Cf-2*
eLRR-TrD-Kinase		Rice	*Xa21*
LRR-TrD-PEST-ECS		Tomato	*Ve1, Ve2*
eLRR-CC		*Arabidopsis*	*RPW8*
TIR-NBS-LRR-NLS-WRKY		*Arabidopsis*	*RRS1-R*
Enzymatic R-genes		Tomato	*Pto*

Ijaz *et al.*,2019

Plant disease resistance

R genes

Dicots and monocots genomes

1. Many studies show that while both *TIR-NBS-LRR* and *CC-NBS-LRR* genes are found in dicot genomes,
2. Only *CC-NBS-LRR* genes are found in monocot genomes.

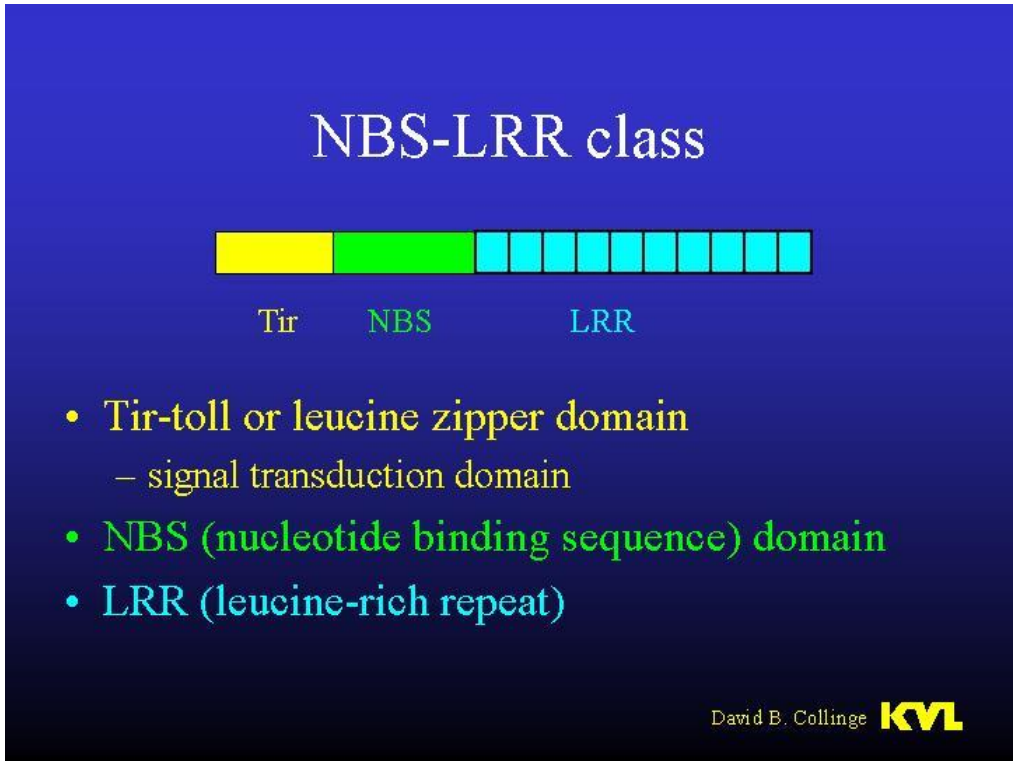
- *NBS-LRR* genes are evolutionary conserved components dedicated to plant innate immunity.

TIR=Tir toll, leucine zipper or coil coil domain
NBS= Nucleotide-binding (NB) sequence domain
LRR= leucine rich repeat domain

Plant disease resistance

R genes

NBS-LRR class or NBS-LRR family



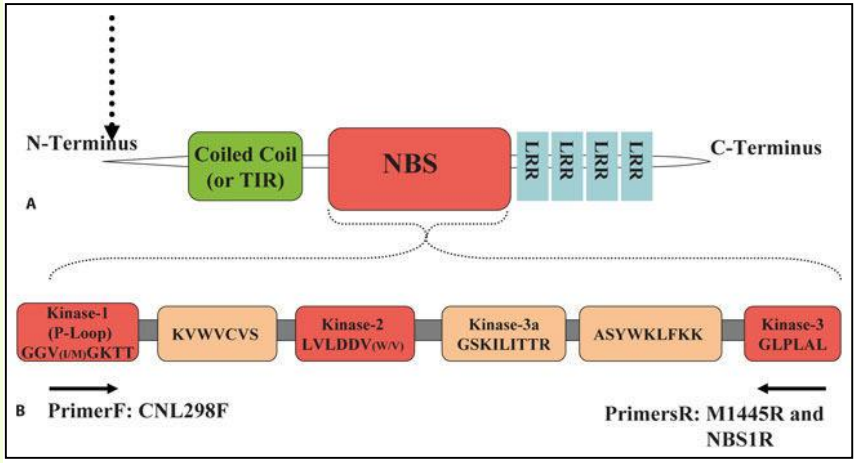
The Tir toll, leucine zipper domain can also be called coil-coil depending on origin.

Plant disease resistance

NB-LRR: The main R-genes

PCR analysis

- Nucleotide-binding site-leucine-rich repeat (NBS-LRR) resistance genes are the largest class of plant resistance genes which play an important role in the plant defense response.
- These genes are better conserved than others and function as a recognition-based immune system in plants through their encoded proteins.



TIR=Tir toll, leucine zipper or coil coil domain.
NBS= Nucleotide-binding (NB) sequence domain.
LRR= leucine rich repeat domain.

Chandrakanth *et al.*,2020

Plant disease resistance

Characteristic structural features

NBS-LRR class or NBS-LRR family

- NBS-LRR proteins are some of the largest proteins known in plants, ranging from about 860 to about 1,900 amino acids.
- They have at least four distinct domains joined by linker regions:

1. a variable amino-terminal domain,
2. the NBS domain,
3. the LRR region, and
4. variable carboxy-terminal domains.

McHale *et al.*,2006

Plant disease resistance

R genes

Functions of the NBS domains

- The NBS domain was defined as a region of ~300 amino acids containing several motifs.

1. NBS domain is responsible for binding and hydrolysis of ATP and GTP during plant disease resistance.
2. NBS domain function in plant defense signaling.
3. It is generally accepted that the primary function of the highly conserved NBS domain is to control the signal transduction through conformational change.
4. Recent studies suggest that the NBS region also might play a role in the establishment of resistance specificity.

Arya *et al.*,2014; Die *et al.*,2018

Plant disease resistance

R genes

Functions of the NBS domains

- Mutations in the NBS domain of *RPS2* destroy (abolished) the ability to induce HR in the presence of AvrRPT2.

1. Therefore NBS domains appear to be essential for R protein function.

Arya *et al.*,2014;..

Plant disease resistance

R genes

Functions of the LRR domains

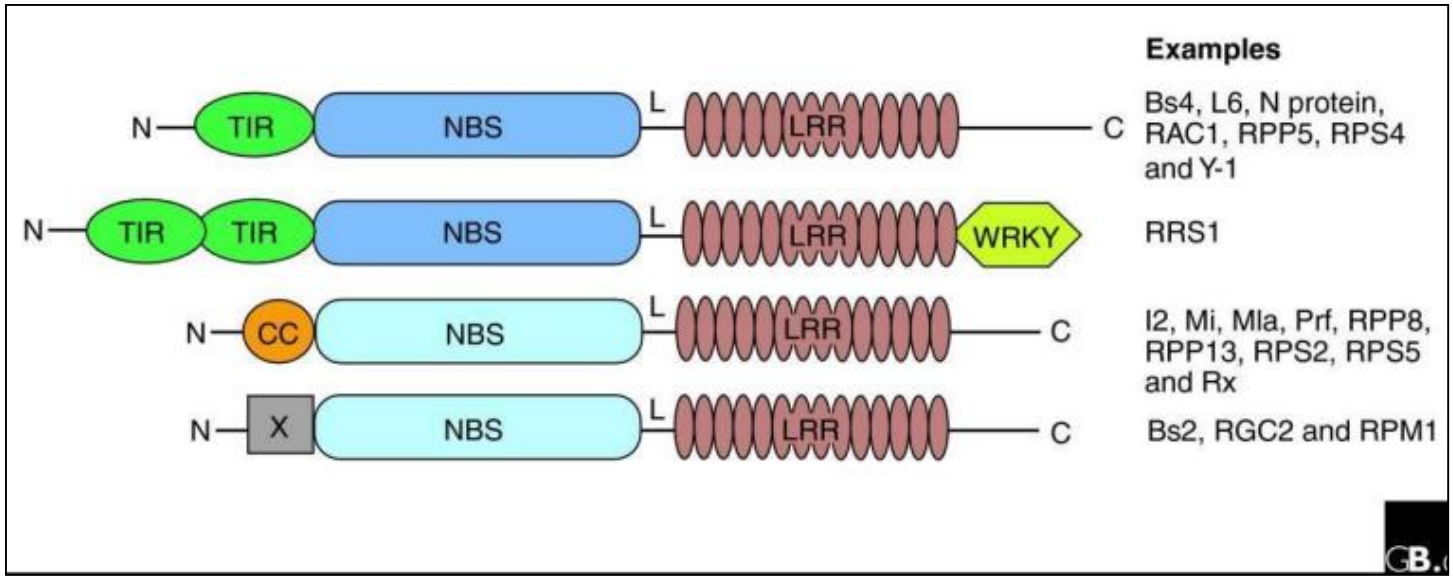
1. LRRs can mediate protein-protein (Avr-R) interactions.
2. Also recognition specificity resides largely in the LRRs.

- It has been suggested that different regions within one NB-LRR protein co-evolved and that intramolecular interaction is required for signal perception and transduction.
- Minor alterations in the LRR domain of e.g. RPS2, RPM1 and N result in a lack of HR activation upon infection by an avirulent pathogen.

Plant disease resistance

R genes

The major domains of NBS-LRR proteins (family)

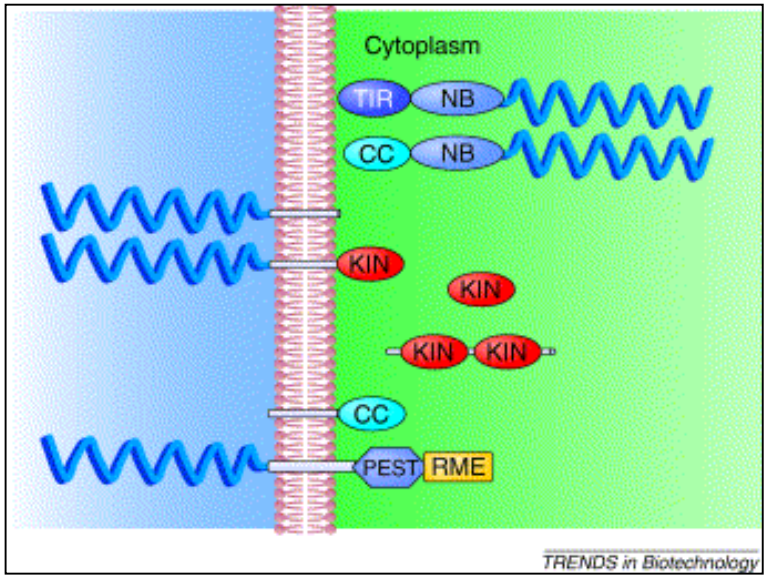


N, amino terminus; TIR, Toll/interleukin-1 receptor-like domain; CC, coiled-coil domain; X, domain without obvious CC motif; NBS, nucleotide binding site; L, linker; LRR, leucine-rich repeat domain; WRKY, zinc-finger transcription factor-related domain containing the WRKY sequence; C, carboxyl terminus.

McHale *et al.*,2006

Major families of R proteins

- The majority of R proteins contain tandem leucine-rich repeats (LRRs, depicted in blue), which have a major role in pathogen effector recognition specificity.



Tandem: lined up one behind another, all facing in the same direction.

McDowell and Woffenden,2003

Plant disease resistance

Characteristic structural features

NBS-LRR class or NBS-LRR family

- Proteins containing LRRs include:

1. tyrosine kinase receptors,
2. cell-adhesion molecules,
3. virulence factors, and
4. extracellular matrix-binding glycoproteins.

- These proteins are involved in a variety of biological processes, including:

1. signal transduction,
2. cell adhesion, DNA repair,
3. recombination,
4. transcription,
5. RNA processing,
6. disease resistance,
7. apoptosis, and
8. the immune response.

Blum *et al.*,2020

Classes of resistance genes

1. Based upon their structural domains

- Based on the predicted localisation of the LRR domain, which can be either:

1. intracellular, or
2. extracellular.

- Plants perceive various PAMPs and effectors by *R* genes encoding for cell surface-localized RLPs or RLKs.
- This perception can be:

1. direct, or
2. indirect.

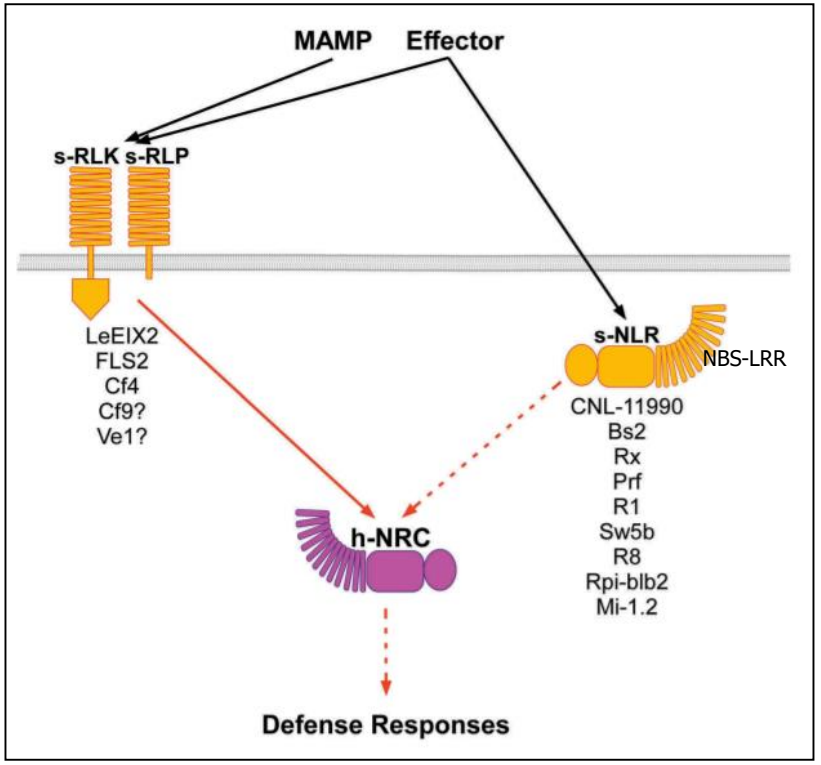
Receptor-like kinase (RLK) and receptor-like protein (RLP) are key pattern-recognition receptors (PRRs) for microbe- and plant-derived molecular patterns that are associated with microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) invasion. RLPs share structural similarity with RLKs but lack a cytoplasmic kinase domain.

Kourelis and van der Hoorn,2018

Classes of resistance genes

PRRs and NB-LRRs in connection with PTI and ETI for plant defense

- Plasma membrane proteins, known as pattern recognition receptors (PRRs), including receptor-like kinases (RLKs) and receptor-like proteins (RLPs) play a fundamental role in perception of PAMPs (PTI) and effectors(ETI).
- Perception of MAMPs or effectors by extracellular or intracellular immune receptors (s-RLK, s-RLP, s-NLR) lead to initiation of immune-signaling by helper NLR (NBS-LRR) proteins called as NRCs, confer immunity to various plant pathogens.



More recently, a new recognition mechanisms have been revealed. In this model NLRs or NBS-LRRs (nucleotide binding leucine rich repeat proteins) can function in pair with a helper NLR proteins called as NRCs, in turn, confer immunity to oomycetes, bacteria, viruses, nematodes, and insects.

Leibman-Markus *et al.*,2018;..

Classes of resistance genes
1. Based upon their structural domains

- The intercellular (I) R proteins also contain:

1. a nucleotide binding site (NBS), and
2. in some cases a leucine zipper (LZ) domain, or
3. a domain with homology to the receptor Toll and the interleukin-1 receptor (TIR).

- In the extracellular (E) proteins the LRR domain is accompanied by:

1. a membrane-spanning region, and
2. in one case by a cytoplasmic protein kinase domain.

Takken and Joosten,2000

Classes of resistance genes
1. Based upon their structural domains

Classes of resistance genes, classified by their structural domains, like transmembrane region (TM), nucleotide binding site (NBS), Toll/interleukin 1 receptor domain (TIR), leucine-rich repeat (LRR) and protein kinase (PK) domain.
The predicted position of the protein (intracellular (I) or extracellular (E) is indicated.

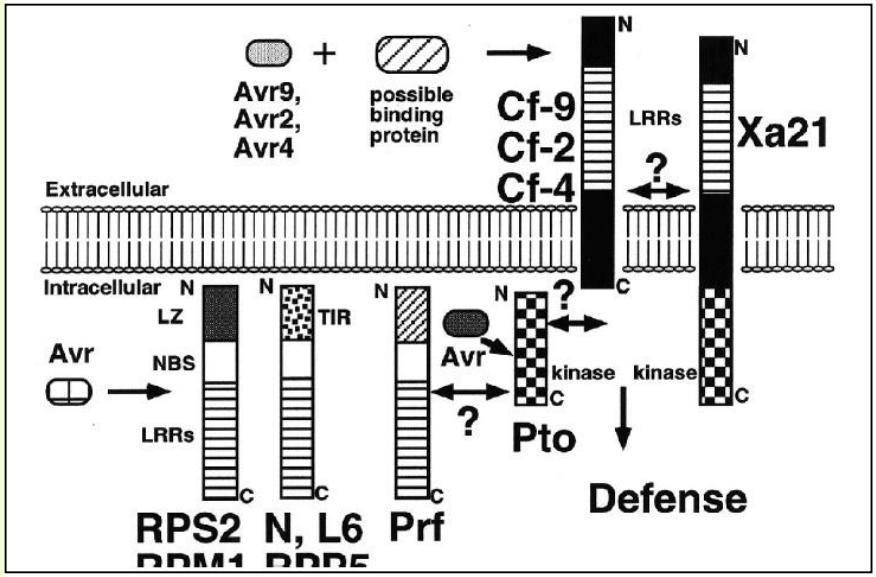
Plant species	*R* gene	Localisation	Structure	Pathogen	Matching gene
Tomato	*Prf*	I	LZ–NBS–LRR	*Pseudomonas syringae* pv. *tomato*	*AvrPto*
Arabidopsis	*RPS2*	I	LZ–NBS–LRR	*Pseudomonas. syringae* pv. *tomato*	*AvrRpt2*
Arabidopsis	*RPM1*	I	LZ–NBS–LRR	*Pseudomonas syringae* pv. *maculicola*	*AvrRpm1, avrB*
Arabidopsis	*RPS5*	I	LZ–NBS–LRR	*Pseudomonas syringae* DC3000	*AvrPphB*
Arabidopsis	*RPP8*	I	LZ–NBS–LRR	*Peronospora parasitica*	*AvrRpp8*
Tomato	*Mi*	I	LZ–NBS–LRR	*Meloidogyne incognita* and *Macrosiphum euphorbia*	
Tomato	*I2c*	I	NBS–LRR	*Fusarium oxysporum*	
Tomato	*I2*	I	NBS–LRR	*Fusarium oxysporum*	
Rice	*Xa1*	I	NBS–LRR	*Xanthomonas oryzae* pv. *oryzae*	
Rice	*Pib*	I	NBS–LRR	*Magnaporthe grisea*	
Potato	*Rx*	I	NBS–LRR	Potato virus X	Coat protein
Potato	*Gpa2*	I	NBS–LRR	*Globodera rostochiensis*	
Wheat	*Cre3*	I	NBS–LRR	*Heterodera avenae*	
Pepper	*Bs2*	I	NBS–LRR	*Xanthomonas campestris*	*AvrBs2*
Corn	*Rp1-D*	I	NBS–LRR	*Puccinia sorghi*	
Rice	*Pi-ta*	I	NBS–LRR	*Magnaporthe grisea*	*AvrPITA*
Barley	*Mla*	I	NBS–LRR	*Erysiphe graminis*	
Tobacco	*N*	I	TIR–NBS–LRR	Tobacco mosaic virus	Replicase
Arabidopsis	*RPP1, 10, 14*	I	TIR–NBS–LRR	*Peronospora parasitica*	
Flax	L^6 L^{l-12}	I	TIR–NBS–LRR	*Melampsora lini*	
Flax	*M*	I	TIR–NBS–LRR	*Melampsora lini*	
Arabidopsis	*RPP5*	I	TIR–NBS–LRR	*Peronospora parasitica*	
Arabidopsis	*RPS4*	I	TIR–NBS–LRR	*Pseudomonas syringae* pv. *pisi*	*AvrRps4*
Rice	*Xa21*	E	LRR-TM-PK	*Xanthomonas oryzae* pv. *oryzae*	
Tomato	*Cf-2*	E	LRR–TM	*Cladosporium fulvum*	*Avr2*
Tomato	*Cf-4*	E	LRR–TM	*Cladosporium fulvum*	*Avr4*
Tomato	*Hcr9-4E*	E	LRR–TM	*Cladosporium fulvum*	*Avr4E*
Tomato	*Cf-5*	E	LRR–TM	*Cladosporium fulvum*	*Avr5*
Tomato	*Cf-9*	E	LRR–TM	*Cladosporium fulvum*	*Avr9*
Sugar beet	$HS1^{pro-1}$	E	LRR–TM	*Heterodera schachtii*	

Takken and Joosten,2000

The *R* genes

Extracellular and intracellular pathogen recognition mechanisms

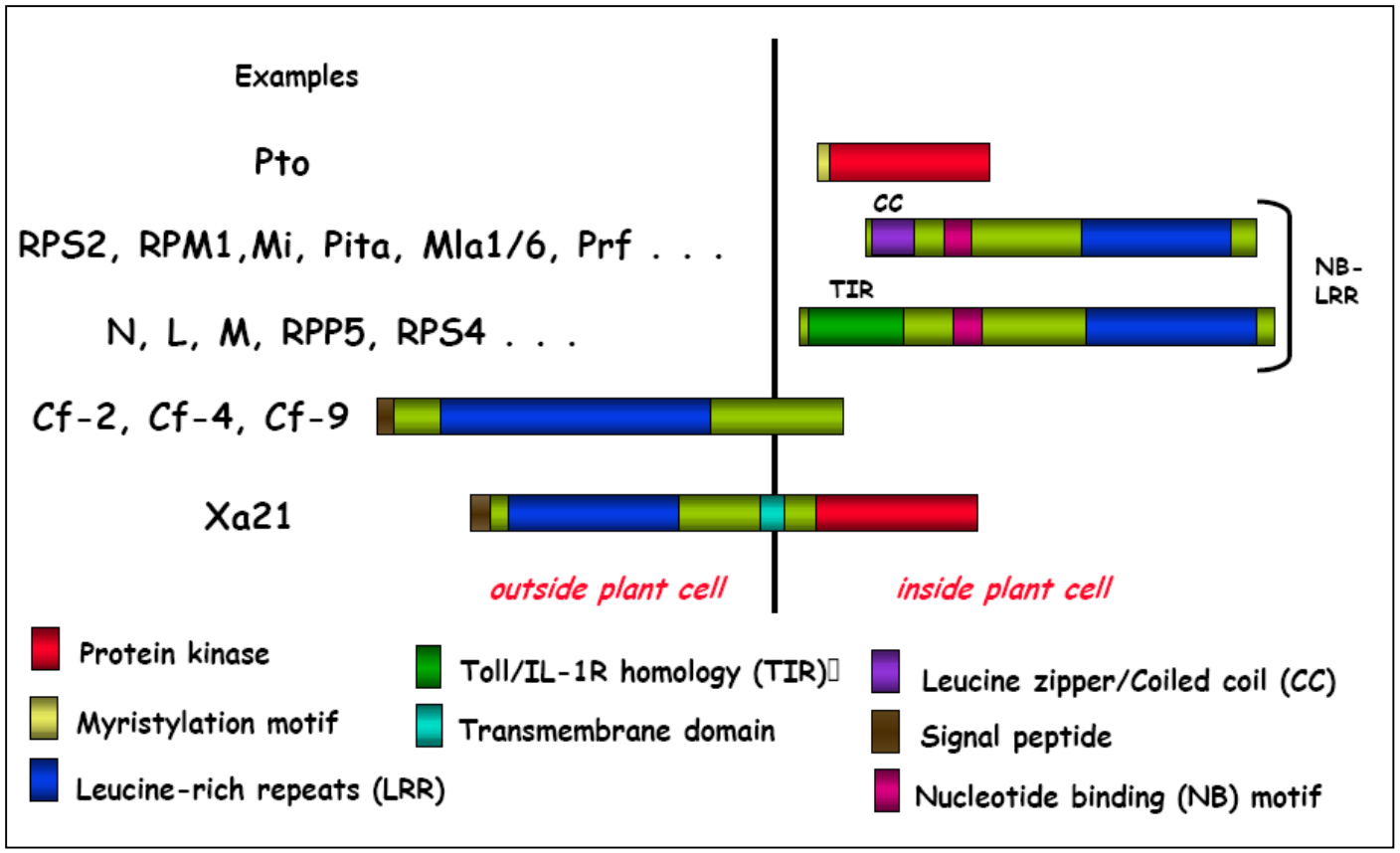
- All R proteins contain a leucine-rich repeat (LRR) domain.
- TIR domain typically code for a coiled-coils structure near their N terminus, sometimes in the form of a leucine zipper.



Hammond-Kosack and Jones,1997

The *R* genes

Features of the 5 major classes of R proteins



Myristylation is co-translational (during translation) protein modification. It often occurs in glycine residues but also on other amino acids. It plays a vital role in membrane targeting and signal transduction in plant responses to environmental stress.

Multiple strategies for pathogen perception by plant immune receptors

The genetics of compatibility and incompatibility

See also Bacterial Pathogenesis-Part 2

Gene-for-gene resistance

Immune receptors

Direct and indirect interactions

- Plants have large numbers of immune receptors in all cells.
- Two main immune receptors are:

1. **Direct interactions** by pattern recognition receptors (PRRs) lead to **PAMP triggered immunity (PTI)**, and
2. **Indirect interactions** by receptors coded by resistance (*R*) genes lead to **effector triggered immunity (ETI)**. This further divided into direct and indirect interactions.

Borrelli *et al.*,2018;..

Gene-for-gene resistance

Immune receptors

Direct and indirect interactions

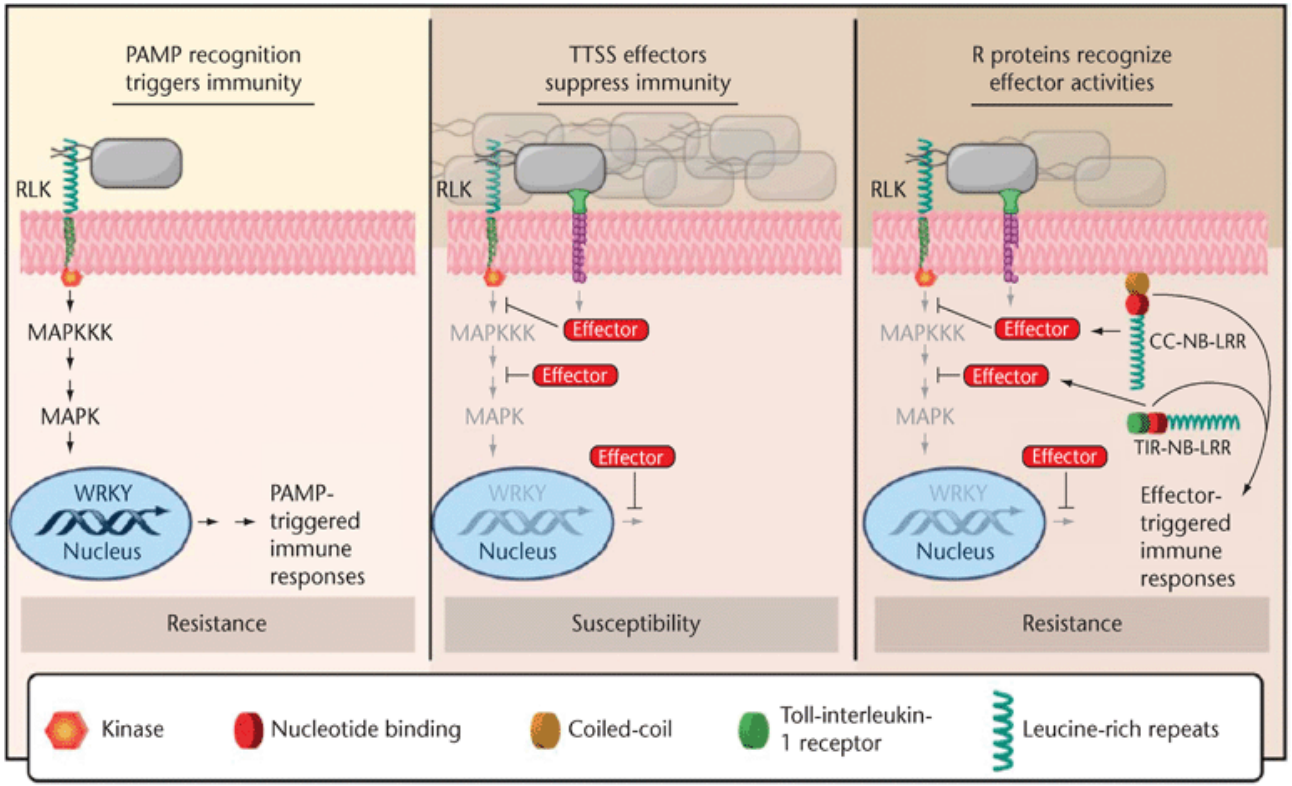
1. PAMP (pathogen-associated molecular patterns)-triggered immunity (PTI) involves direct recognition of conserved PAMPs and does not involve HR.
2. Effector-triggered immunity (ETI) by many *R* genes confer direct and indirect recognition of pathogen-derived effectors, which often involves the hypersensitive response (HR), a type of programmed cell death.

Kourelis and van der Hoorn,2018

Gene-for-gene resistance

Immune receptors

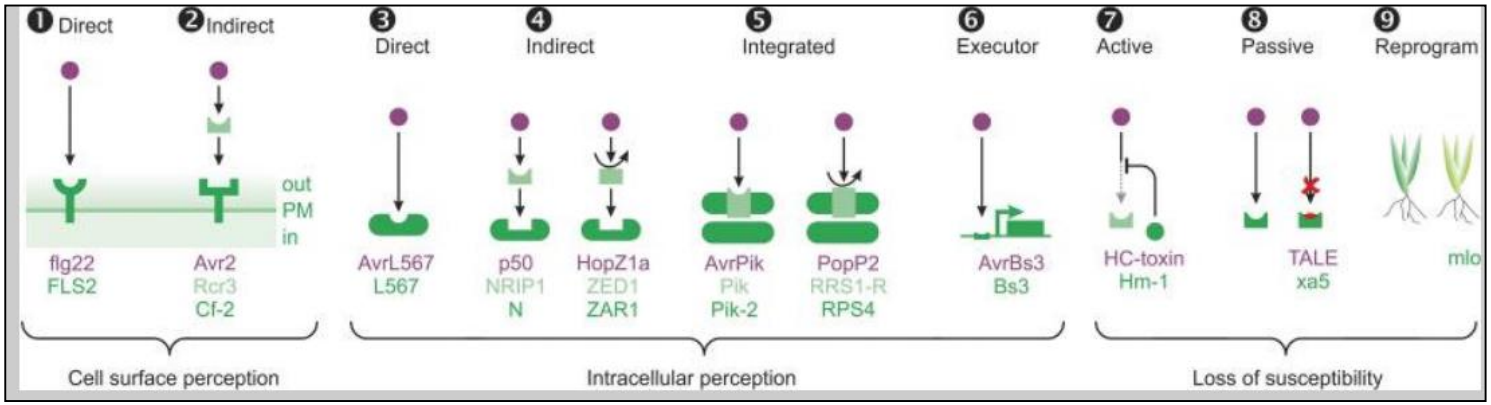
PAMP or effector triggered immunity



Left, recognition (PAMP) triggers basal immunity; Middle, a cocktail of effector proteins is produced and delivered within plant cells via the Type III secretion system; Right, plant resistance proteins (CC-NB-LRR and TIR-NB-LRR here) detect effector activity or the presence of the effector protein, and restore resistance through effector-triggered immune response. RLK: Plant receptor-like protein kinases are transmembrane proteins found in most eukaryotic organisms.

Classes of resistance genes

R genes classified by nine mechanisms



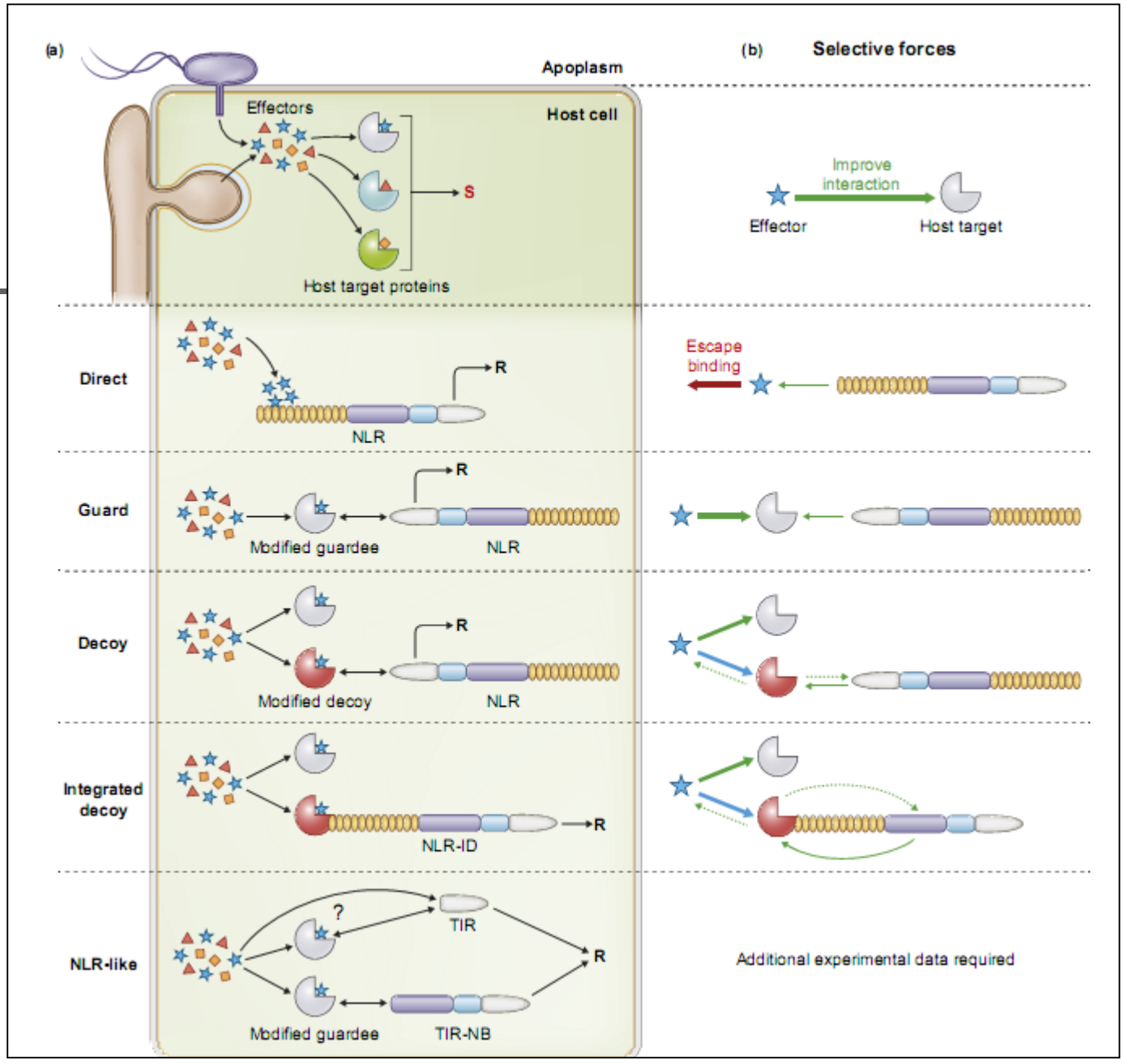
Nine Molecular Mechanisms Underpinning *R* Gene Functions.

Illustration of direct (1) and indirect (2) recognition at the cell surface; four different intracellular perception mechanisms (3–6); and three loss-of-susceptibility mechanisms (7–9).
PAMPs and effectors are colored in purple, indirect receptors in light green, and direct receptors in dark green.

Kourelis and van der Hoorn,2018

Classes of resistance genes

R genes classified by five mechanisms.



NLS: primarily nucleotide-binding, leucine-rich repeat proteins

Stella Cesari,2017

Classes of resistance genes
R genes classified by nine mechanisms

- Recognition of effectors by NLR (NBS-LRR section of R proteins) proteins can either be direct or indirect.

1. In direct effector recognition, direct interaction between an effector and NLR protein is necessary for recognition (Jones & Dangl, 2006).
2. In indirect effector recognition,

- modifications of effector targets (a guardee), or
- mimics of true host targets (a decoy) are sensed by NLR proteins.
- additional atypical domains referred to as 'integrated decoy domains' or 'sensor domains'.

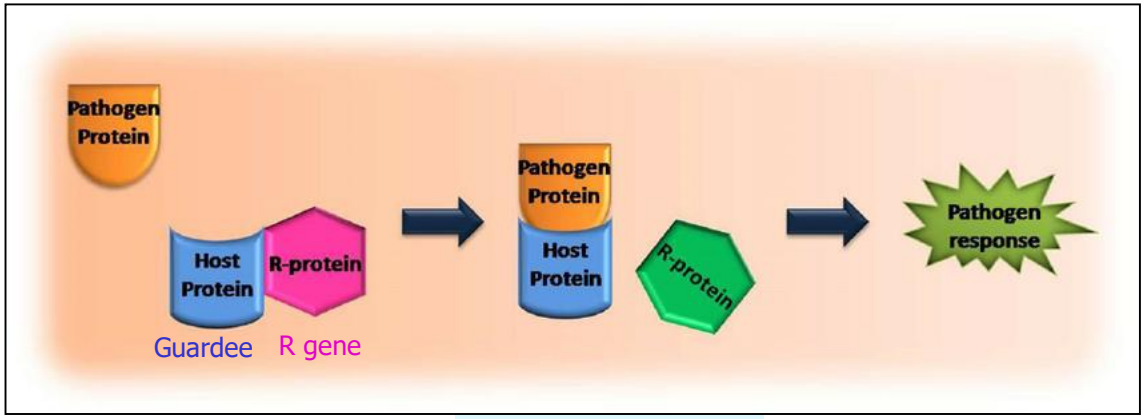
Kroj *et al.*,2016

Gene-for-gene resistance

Indirect interactions of AVR-R proteins

The Guard hypothesis

- The plant R proteins (guard) are associated with the endogenous host protein (guardee) which are common target proteins for the pathogens.
- The interaction of effector pathogen proteins with the host proteins, causes a change in their structure which is then recognized by the guard proteins.
- As a result, a pathogen response signaling cascade is triggered against the microbial evasion.



Gururani,2012

Gene-for-gene resistance

Immune responses

Decoy model

- Modification of the guard model, known as the decoy model, was proposed.
- Decoy would be solely involved in effector perception.
- A decoy as an accessory protein mimics the operative effector target without contributing pathogen fitness in the absence of its cognate R protein.

Borrelli *et al.*,2018;..

Gene-for-gene resistance

Indirect interactions of AVR-R proteins

Decoy Model

- The decoy is a duplicated guardee without a function in plant immunity.
- Its sole role is to trap effectors, thereby activating the immune signaling cascade.

Gene-for-gene resistance

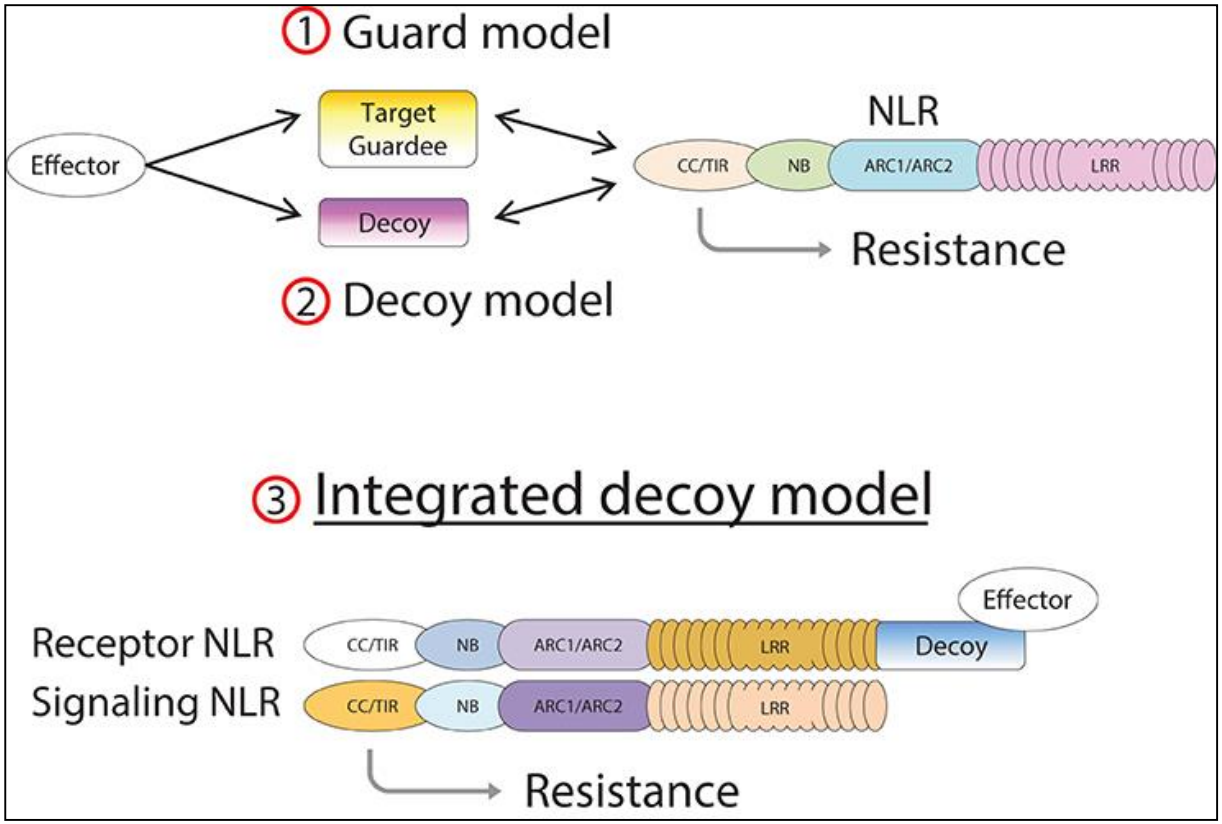
Indirect interactions of AVR-R proteins

Decoy Model

- Alternatively the decoy may be integrated into the structure of the receptor component of an NLR (NBS-LRR of R proteins) pair, allowing AVR recognition by direct binding.
- Recognition of pathogen effector proteins by plant NLR proteins often involves decoy proteins, which mimic effector targets.
- In certain examples such decoys are integrated as fusions into their cognate NLR.

Gene-for-gene resistance

Decoy and integrated decoy hypothesis

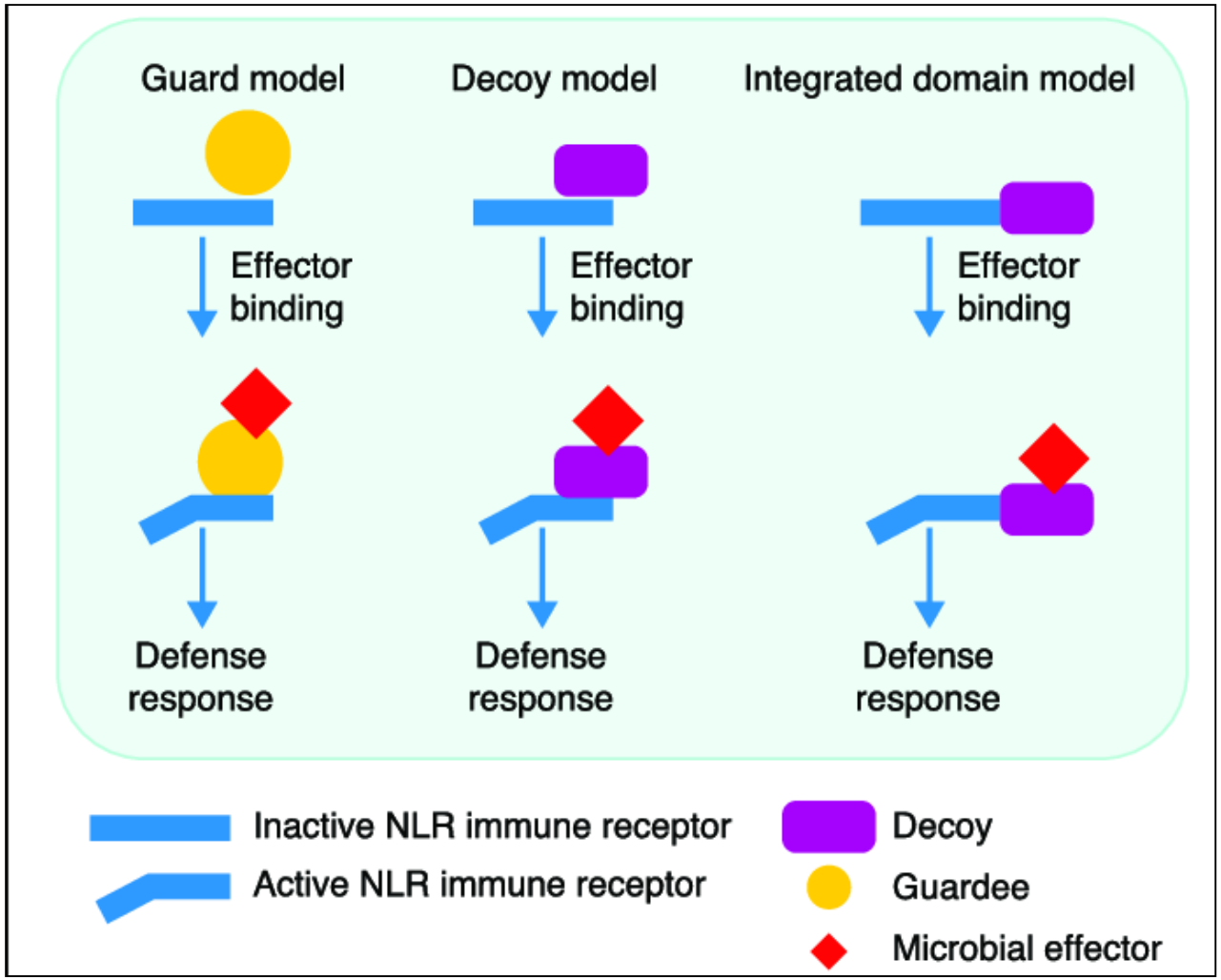


The NLRs or NBS-LRRs (nucleotide-binding, leucine-rich-repeat) form the largest resistance gene family in plants.

Cesari *et al.*,2014

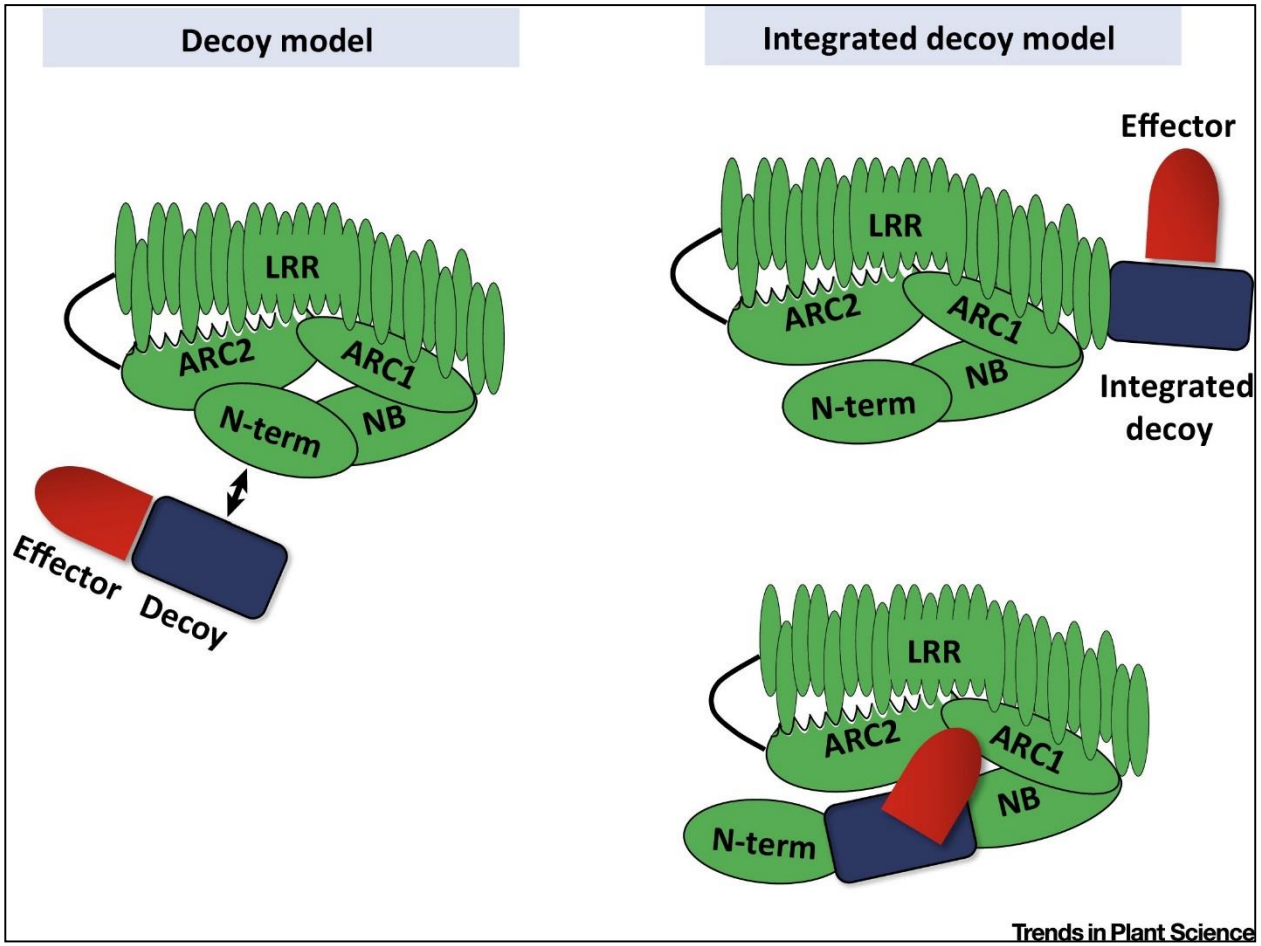
Gene-for-gene resistance

Decoy and integrated decoy hypothesis



Gene-for-gene resistance

Decoy and integrated decoy hypothesis



Controlling *R* genes
"on"/ "off" switch

- R proteins must be tightly regulated in order for plants to maintain vigilance against possible microbial invasion while avoiding unnecessary activation of defenses in the absence of pathogens to minimize the cost associated with R protein expression (Tian *et al.*,2003).
- There exist at least two levels of regulation:

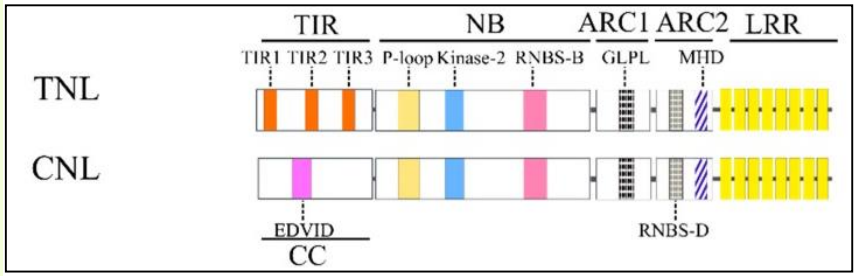
1. mRNA, and
2. Protein.

Some *R* genes are expressed at very low levels prior to pathogen challenge, and are induced to higher levels by inoculation of the cognate pathogens, suggesting that there must be a pathogen-responsive "off" and "on" switch controlling those *R* genes at the transcription level.

Model for the switch function of NBS-LRR proteins

On, off and intermediate state

- Between the NBS and LRR domains exists a region called the ARC domain.
- This ARC domain can be further divided into ARC1 and ARC2 subdomains.
- The ARC domain, together with the NBS domain, forms a region for nucleotide binding.



The NLRs or NBS-LRRs (nucleotide-binding, leucine-rich-repeat) form the largest resistance gene family in plants with lineage-specific contingents of TNL, CNL and RNL subfamilies.

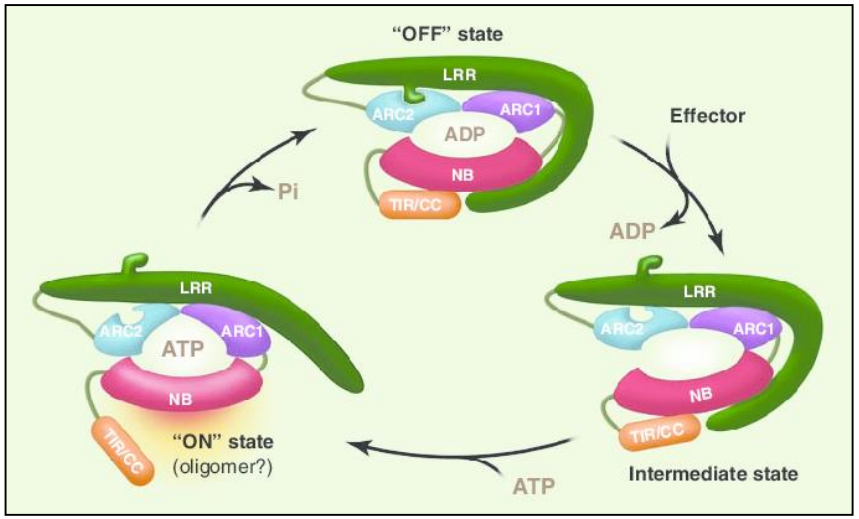
The NLRs or NBS-LRRs (nucleotide-binding, leucine-rich-repeat) form the largest resistance gene family in plants. The NB-ARC domain is a novel protein motif shared by important plant and animal proteins whose activation results in cell death.

Sekhwal *et al.*,2015

Model for the switch function of NBS-LRR proteins

On, off and intermediate state

- In the absence of a pathogen an NB-LRR protein resides in its resting (ADP) state (OFF or resting state), in which the LRR stabilizes the closed conformation.
- The presence of an effector (Avr) affects the LRR domain, which induces a conformational change in the NB-ARC domain that allows the release of ADP.
- ATP binding subsequently triggers a second conformational change in the N-terminal effector domain, releasing its signalling potential.
- The ATPase activity of the protein attenuates the signalling response and returns the protein to its resting state.

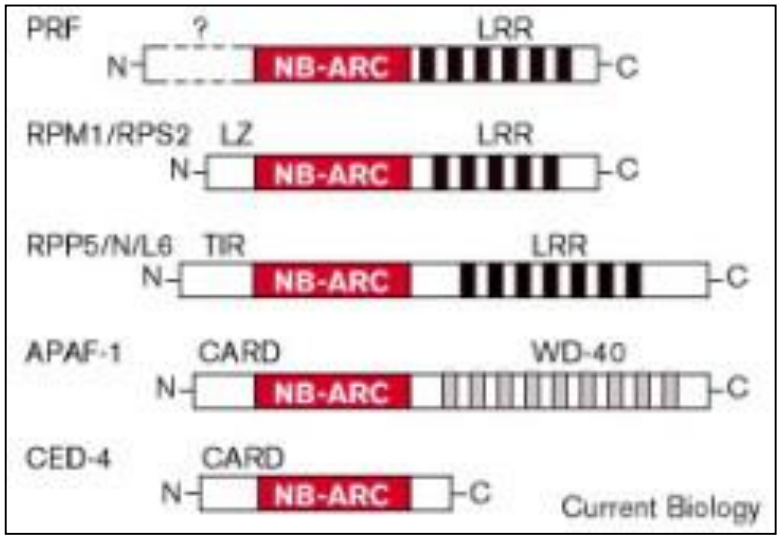


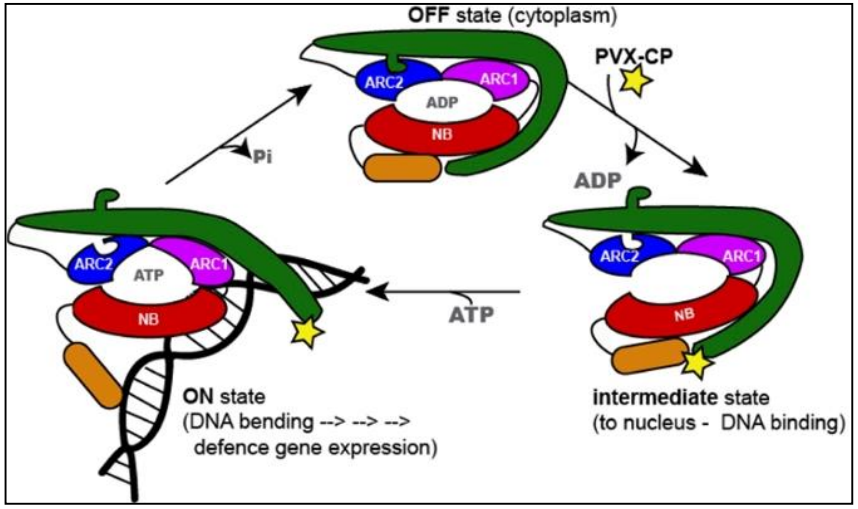
ADP (adenosine diphosphate) and **ATP** (Adenosine tri-phosphate). ATP is the energy currency of the cell. Hydrolysis of ATP could return the protein to its resting state.

Takken *et al.*,2006 & 2009

Model for the switch function of NBS-LRR proteins

On, off and intermediate state





Left: The NB-ARC domain: a novel signalling motif shared by plant resistance genes. Right: The NB-ARC domain of NB-LRRs functions as a molecular switch wherein the ADP-bound state represents the "off" and the ATP-bound state as the "on" state.

Erik *et al.*,1998;Takken *et al.*,2006

Genetic Engineering

Plant transformation/transgenic plant production

1. *R* genes
2. With *NPR1/* PRs genes
3. Gene silencing
4. Enhancing levels of reactive oxygen species
5. With genes encoding enzymes which degrade bacterial toxins
6. Modifying the phytoalexin response
7. Anti-microbial proteins (AMPs)/antifungal proteins (AFP)
8. Plantibody-mediated resistance against plant diseases

Plant biotechnology

Plant transformation/transgenic plant production

An approach to identify and design the novel genes

- Plant biotechnology as a new era for plant pathology and plant protection is directed at the design of novel genes that will enable transformed plants to inactivate or resist key pathogenicity factors.
- These genes can be obtained from different sources and engineered into plants to enhance resistance to plant pathogens.
- The four major areas of research and application of plant genetic engineering for resistance to plant pathogens consist of:
- Enhancing resistance with plant genes;
- Enhancing resistance with pathogen derived genes (derived from the pathogens themselves);
- Enhancing resistance with antimicrobial proteins;
- Enhancing resistance with plantibodies.

Fermin-Muñoz *et al.*,2000

Transgenic approach

R genes mediate resistance to bacterial, fungal, viral, and nematode pathogens

- Plants have their own networks of defense against plant pathogens that include a vast array of proteins and other organic molecules produced prior to infection or during pathogen attack.
- Recombinant DNA technology allows the enhancement of inherent plant responses against a pathogen by either using:

1. Single dominant resistance genes not normally present in the susceptible plant (Keen,1999) or
2. By choosing plant genes that intensify or trigger the expressions of existing defense mechanisms.

The *avr* genes
Impractical use in transgenic plants

- Although it would be tempting to express an effector gene in transgenic plants in order to create bacterial disease resistance by the elicitation of the HR, there are a number of reasons why this should not be considered for immediate practical use. e.g.

1. Effectors determine race-specific plant recognition events, thus, a whole set of genes would be necessary to provide protection against a wide range of pathogenic races.
2. Since the function of most effectors is not clear yet, expression of a single transgene might not induce the HR as has already been observed when bacteria harbouring various effector genes were infiltrated into plant leaves.

Genetic Engineering Plants

Successful example of engineering *R* genes

1. *R* genes- mediated resistance

R genes-mediated resistance

- With the availability of cloned resistance genes there is now the opportunity to transfer them speedily into genotypes in which the gene is lacking.
- Pathosystem-specific plant resistance (*R*) genes have been cloned from several plant species.
- These include *R* genes that mediate resistance to bacterial, fungal, viral, and nematode pathogens.
- Many of these *R* gene products share structural motifs, which indicate that disease resistance to diverse pathogens may operate through similar pathways.

Identification of resistance (*R*) genes through genetics

- Plant breeders have successfully introduced disease resistance through introgression (breeding) of foreign *R*-genes.
- However, pathogen races quickly evolve that lack the cognate *avr* genes.
- The adaptive ability of pathogens has limited the durability of most *R*-gene-based resistance.
- Sources of new *R*-genes are sought.
- These may be introduced into commercial crop lines through introgression (breeding), or by genetic engineering.
- The latter requires molecular cloning of *R*-genes.

Genetic engineering plant
Cloning of *R*-genes

- The cloning of the first resistance gene, *Pto*, which confers resistance of tomato to *Ps. syringae* pv. *tomato* was reported in November 1993 (Martin *et al*.,1993).
- Since then, more than 30 resistance genes, which are effective against fungi, bacteria, viruses and nematodes, have been cloned and sequenced.
- At least 11 plant resistance genes conferring resistance to bacterial diseases have been cloned to date.

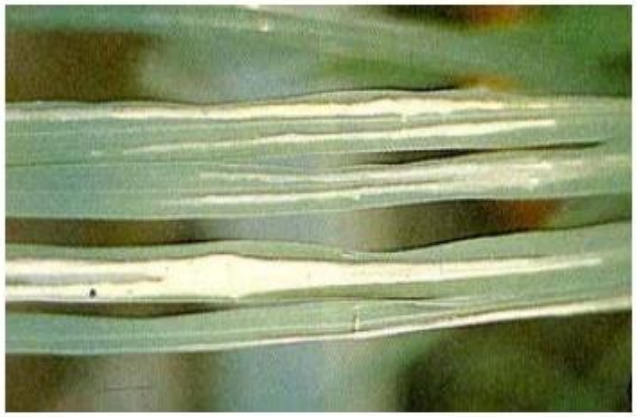
Improved disease resistance in transgenic plants over expressing defense related *R* genes

R Gene	Identity	Transgenic host plants	Effects observed in transgenic plants
Hml	NADPH-dependent HC toxin reductase	Maize	Controls resistance to the fungus *Cochliobolus carbonum* race 1
N	Interleukin-1 mammalian like protein	Tobacco	Confers resistance to tobacco mosaic virus (TMV)
Cf-9	Elicitor	Tobacco	Resistance to *Cladosporium fulvum*
Flax L^6		Maize	Resistance to *Melampsora lini*
PTO	Serine-threonine protein kinase	Tomato	Confers resistance to *Pseudomonas syringae* pv tomato
RPS2	Leucine-rich repeat protein	*Arabidopsis*	Confers resistance *to Pseudomonas syringae* pv tomato
RPM1	Leucine Zip-like protein	*Arabidopsis*	Confers resistance to *Pseudomonas syringae* pv tomato
Xa7		Rice	Confers resistance to *Xanthomonas oryzae* pv oryzae race 6
Pita	Neutral zinc metalloprotease	Rice	Confers resistance to *Magnaporthe grisea*
Rar1	Homologous to the yeastSGT1 protein: positive regulator of E3 ubiquitin ligase	Barley	Confers resistance to powdery mildew
EDS1	Lipase like protein	*Arabidopsis*	Mediates the down stream signaling of known TIR-type
PAD4	Lipase like protein	*Arabidopsis*	Mediates the down stream signaling of known TIR-type

R genes diverse pathogens encode proteins with leucine-rich repeats (LRRs)

N gene / tobacco mosaic virus

Cf9 gene / fungal leaf mold



Xa21gene / bacterial blight

Mi gene / root-knot nematode

Pto
The first cloned *R* gene

- The first cloned *R* gene was *Pto*, which confers resistance to tomato against the bacterial pathogen *Pseudomonas syringae* pv.*tomato*.
- Models for R protein function.
- The analysis of Pto led to models for *R* gene product function:
- The Pto protein requires an additional NB-LRR protein called Prf (The Guard Hypothesis) in order to mediate resistance to *P. syringae* pathogens carrying the AvrPto gene.

The most successful example of engineering R genes

***Pto* Resistance gene:**

1. When the first cloned disease-resistance gene *Pto* (a tomato resistance gene against *P. syringae* pv. *tomato*) was found to function both in *Nicotiana tabacum* and *N. benthamiana* suggesting that disease resistance functions are conserved in a wide range of plant species.
2. *Pto*-overexpressing plants show resistance not only to *P. syringae* pv.*tomato* but also to *X. vesicatoria* and to the fungal pathogen *Cladosporium fulvum* (Mysore *et al*.,2003).

Genetic engineering plant

Pto Resistance gene

Against *P. syringae* pv. *tomato*

- Over expression of *Pto* in tomato under control of the cauliflower mosaic virus (CaMV) 35S promoter has been shown to activate defense responses in the absence of pathogen inoculation.
- Therefore, *Pto* genes are considered as potential candidates to protect plants against pathogens.

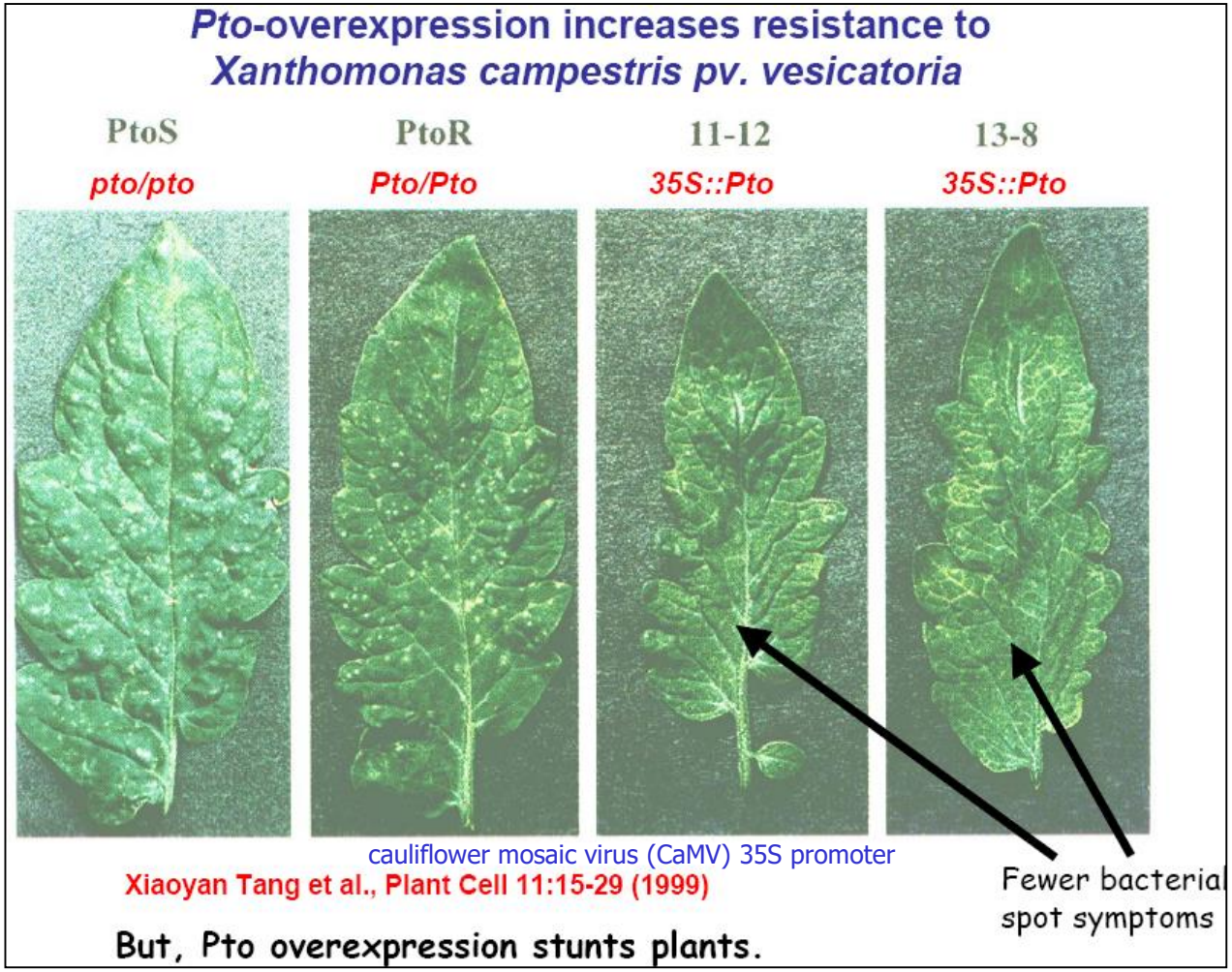
Genetic engineering plant
Pto Resistance gene

- Microsocopic examination showed that the transgenes had small necrotic areas in the palisade mesophyll cells resembling the hypersensitive response induced by incompatible pathogens.
- Furthermore, the plants accumulated salicylic acid and increased levels of pathogenesis-related proteins (PRs).
- It therefore seems that overexpression of a resistance gene in the absence of the corresponding avirulence gene of the pathogen can give a phenotype with broad-based resistance.

Genetic engineering plant

Pto Resistance gene

Against *Xanthomonas vesicatoria*



Genetic engineering plant

*Xa*10 Resistance gene

Xanthomonas oryzae pv. *oryzae*

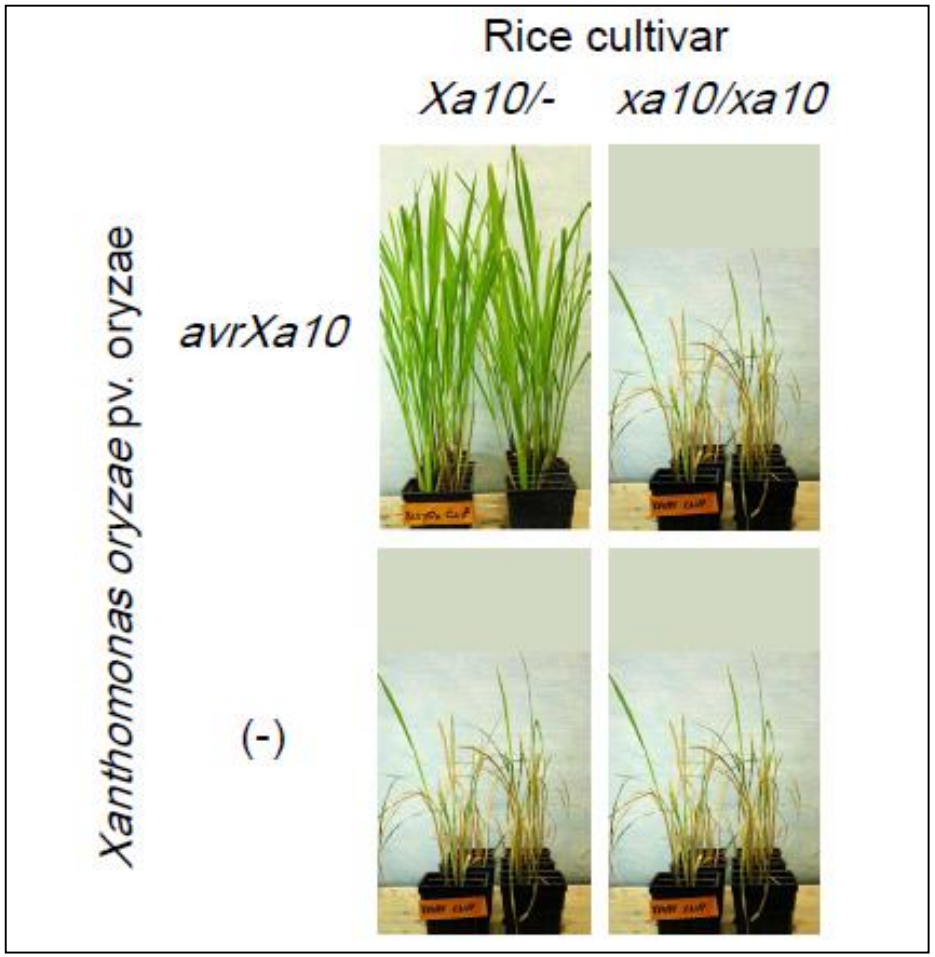
- Cultivar/race specificity is governed by Avr-R gene interaction which mediates resistance.
- For example, rice cultivar carrying resistance gene Xa10 can develop resistance against Xoo carrying avirulence gene avrXa10.
- This is incompatible interaction since the pathogen cannot cause disease in a resistance plant.
- However, even either Xa10 or avrXa10 is lacked, the interaction results in disease development which is compatible interaction.

Disease resistance

Cultivar/race specificity is governed by *avr-R* gene interaction

Xanthomonas oryzae pv. *oryzae*

Rice cultivar carrying resistance gene Xa10 can develop resistance against Xoo carrying avirulence gene avrXa10.



Bogdanove

Genetic Engineering Plant

Bs2 Resistance gene

Confers resistance to strains of *X. vesicatoria*

- The *Bs2* resistance gene of pepper specifically recognizes and confers resistance to strains of *X. vesicatoria* (XV) that contain the corresponding bacterial avirulence gene, *avrBs2*.
- Transgenic tomato plants expressing the pepper *Bs2* gene suppress the growth of *X. vesicatoria* (XV).

Genetic Engineering Plant

Bs2 Resistance gene

Confers resistance to strains *X. campestris* pathovars

- The *Bs2* gene of pepper confers resistance to strains *X. campestris* pathovars too.
- The Bs2 gene may be durable in the field and provide resistance when introduced into other plant species.
- Functional expression of Bs2 in transgenic tomatoes has demonstrated that it can be used as a source of resistance in other solanaceous plant species and perhaps in banana, too.

Genetic Engineering Plant

Xa 21 Resistance gene

Confers resistance to *X. oryzae* pv.*oryzae*

- The most successful example of engineering *R* genes in crop plants is definitely the *Xa21* gene from rice, which confers resistance to all races of *Xanthomonas oryzae* pv.*oryzae*.
- The gene was cloned by Song *et al.*,1995 and susceptible plants were transformed biolistically.
- The gene has been introduced into a wide range of susceptible varieties and was repeatedly found to provide resistance under laboratory conditions as well as in the field.

Genetic Engineering Plant

Xa 21 Resistance gene

Confers resistance to *X. oryzae* pv.*oryzae*

- Comparison of genotypes of rice resistant and susceptible to *Xanthomouas oryzae* pv. *oryzae* and a susceptible genotype transformed with the resistance gene Xa21 (all plants were challenged with the pathogen).
- From left to right:
- IRBB21 (resistant),
- IR24 (susceptible),
- TP 309 (susceptible), and
- TP309 (transformed with Xa21).

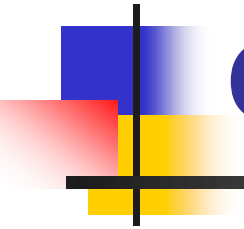
(courtesy of Pamela Ronald, University of California, Davis, USA)

Genetic Engineering Plant

XaPES1 Resistance gene

Confers resistance to *R. solanacearum*

- The *RRS1* gene has recently been identified in *Arabidopsis* at a recessive locus for resistance to *R. solanacearum*, a pathogen of banana.
- This gene that confers resistance to several races of *R. solanacearum* has recently been cloned and found to fall into a specific class of the above NBS-LRR type of *R* genes.
- The gene can be a good candidate for transfer to banana, and testing for resistance to Moko disease caused by *R. solanacearum*.



Cloning of *R*-genes

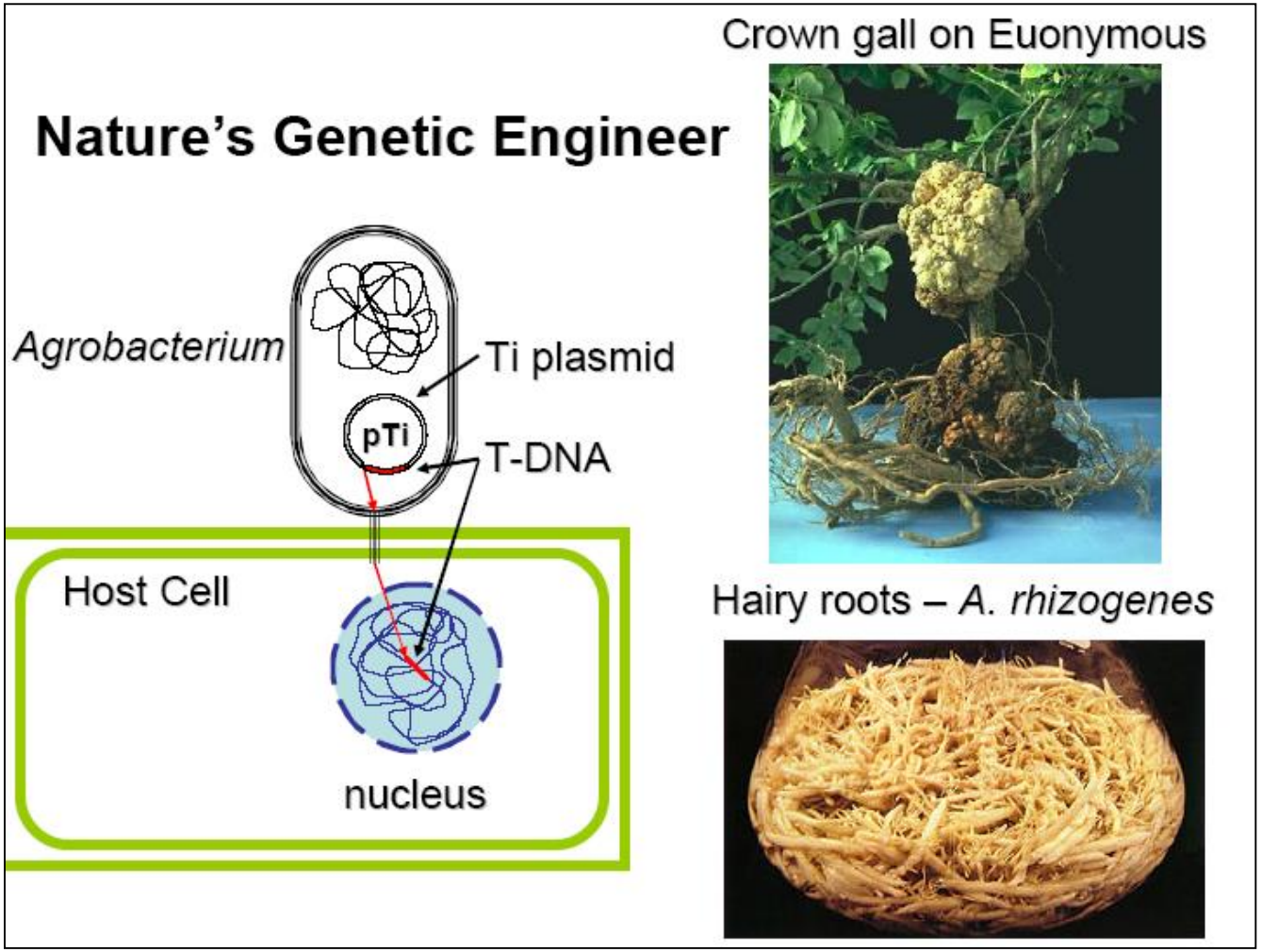
Principles and Methods

Natural genetic engineering

- The realization that plant transformation was not only a possibility but had already occurred in nature was a consequence of the discovery that the symptoms of crown gall and hairy root diseases, caused by *Agrobacterium tumefaciens* and *A. rhizogenes*, respectively, are the result of 'natural genetic engineering'.

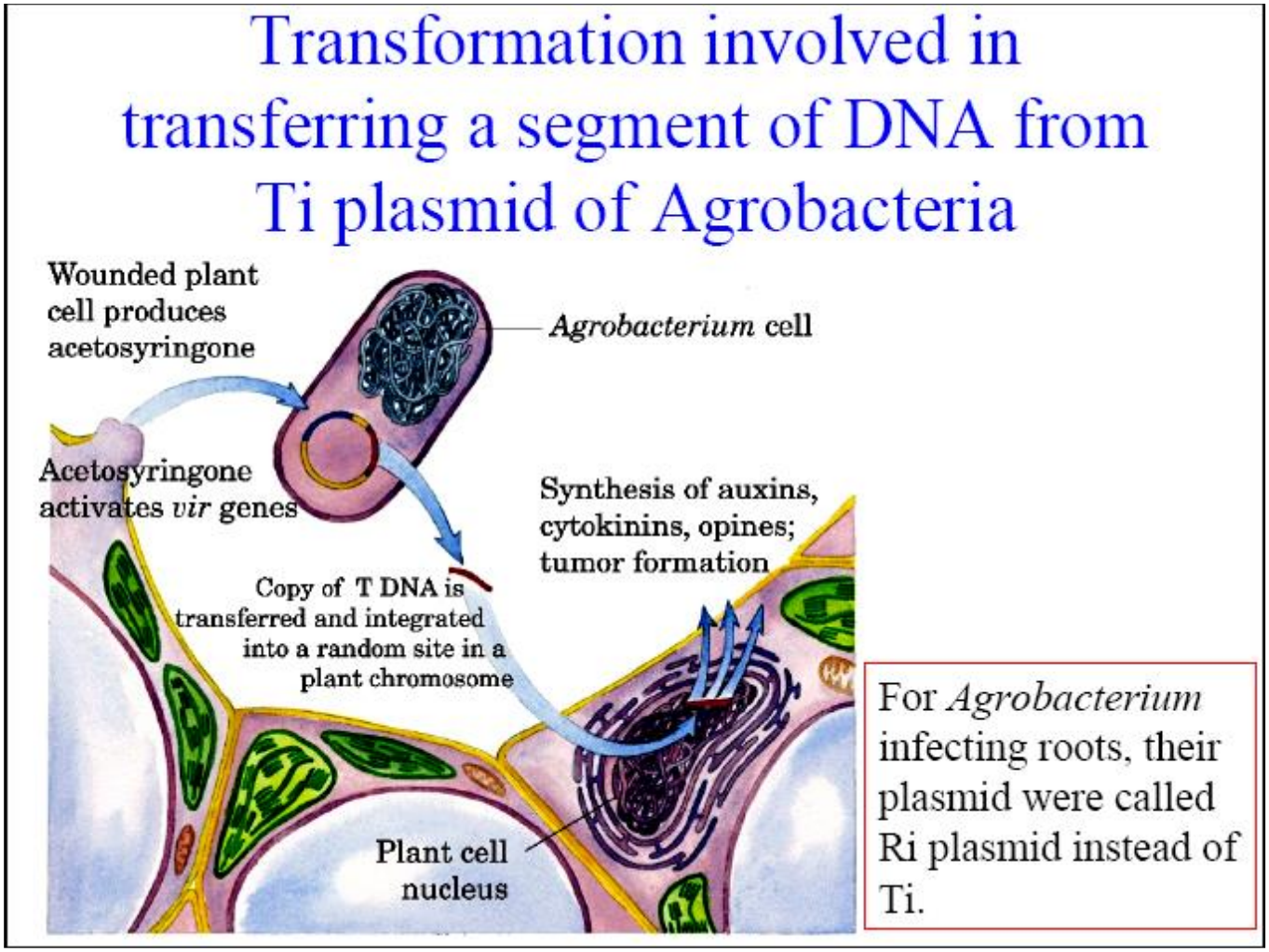
Agrobacterium tumefaciens mediated transformation

A natural tool for plant transformation



Agrobacterium tumefaciens mediated transformation

A natural tool for plant transformation



Engineering strategies
Transformation experiments

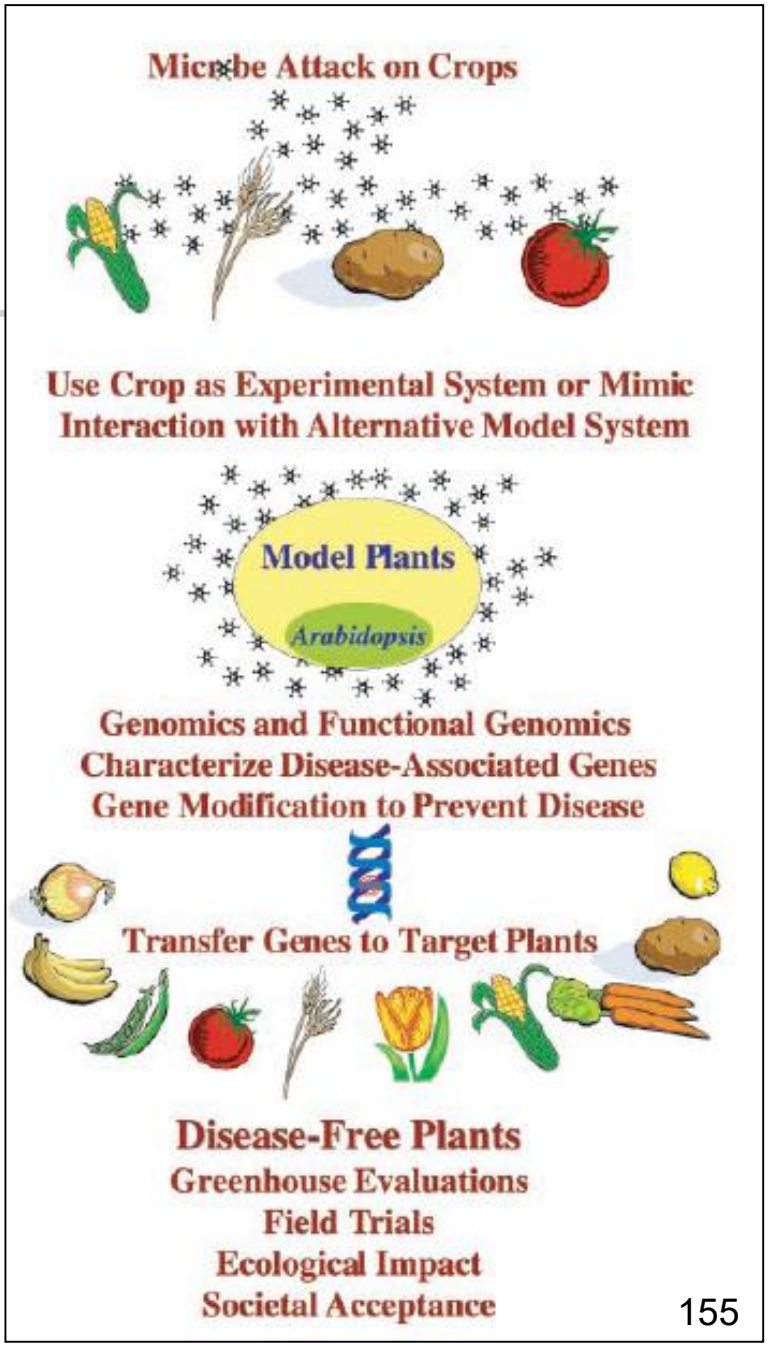
- In principle, many of the engineering strategies outlined below may be applied to control plant diseases.
- However, before transformation experiments can proceed, detailed biochemical and genetic (complete genome sequencing) studies of well-characterized strains will be necessary.
- Initial investigations might focus, for example, on the:
- Mode of action and structure of bacterial toxins, and of extracellular polysaccharides.

The model plant Arabidopsis genome

- The model plant Arabidopsis genome contains around 200 *R* genes that encode proteins with similarity to the nucleotide-binding site and other domains characteristic of many identified plant resistance (R) proteins (Meyers *et al.*,2003).

Arabidopsis plant genome is a molecular research model

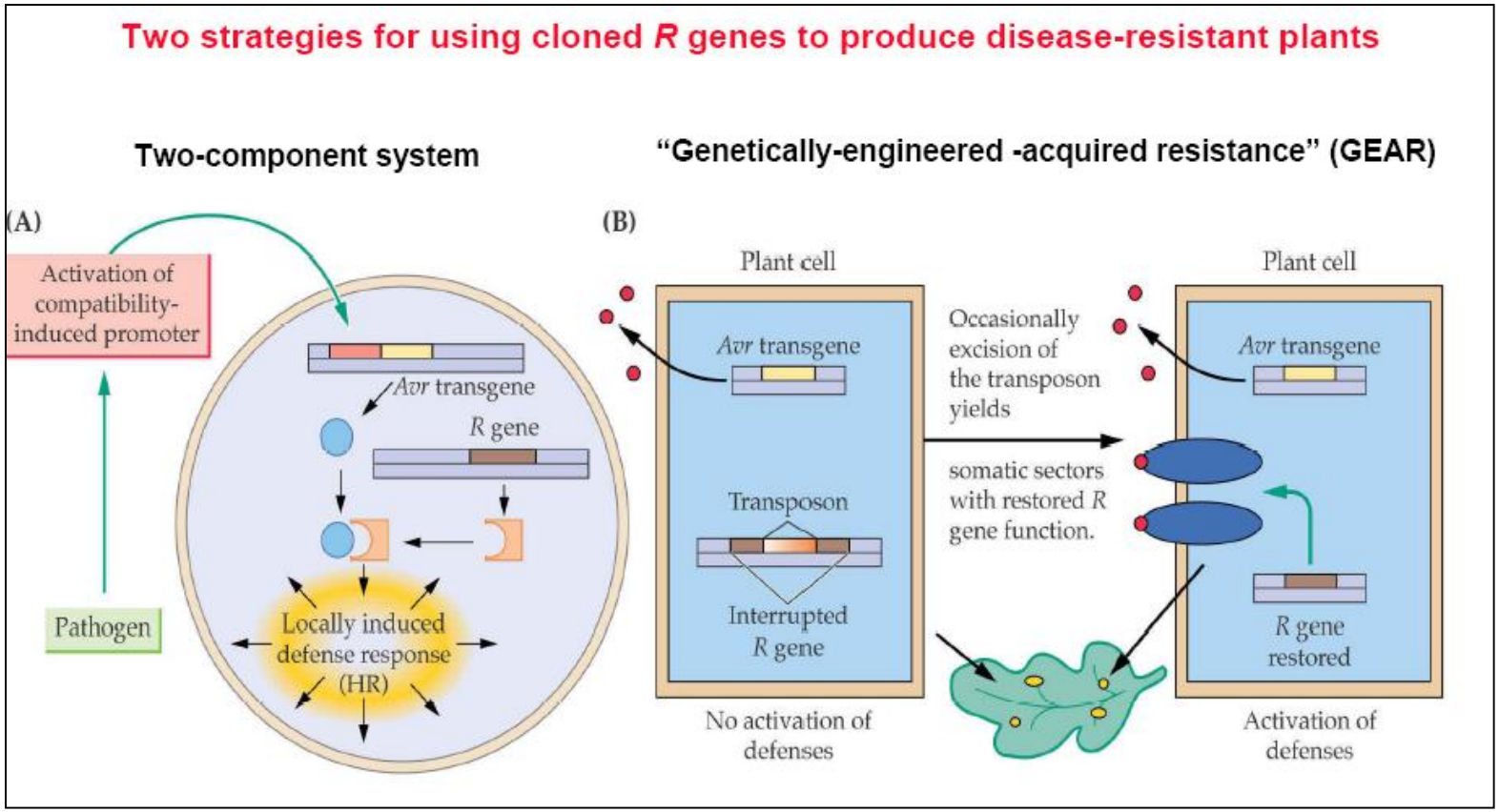
- *Arabidopsis* and other model plants has been explored for many plant-pathogen combinations.
- *Arabidopsis* genome helps to develop durable and broad-spectrum control measures, functional genomics efforts on diverse plant systems will be instrumental.



Engineering plant resistant to pathogens

- The cloning of *R* genes has opened doors for producing disease-resistant crop plants.
- Transgenic plants that resist pathogen attacks can be engineered either by:
- Insertional mutagenesis (*Agrobacterium tumefaciens* mediated transformation).
- Map-based cloning methodology.

Two strategies for using cloned *R* genes to produce disease-resistance plants



1. Insertional mutagenesis method of transformation

Agrobacterium tumefaciens mediated transformation

- The most commonly used DNA molecules for insertional mutagenesis are:
- Transposons,and
- T-DNA from the Ti plasmid.

Transposon tagging

- In this cloning strategy, plants homozygous for known resistance genes are transformed with a transposon.
- They are then crossed with a plant with no known resistance genes to produce F1 progeny which are heterozygous for the resistance allele.

Construction of Ti vectors

- The essential components of the plasmid for successful transformation being a 25 bp border sequence at the right of the T-DNA (i.e. the DNA that is transferred to the plant) and the *vir* region.
- And since the *vir* region can function *in trans*, it need not be on the same plasmid as the T-DNA.
- Therefore, various Ti-vectors (plasmids bearing the T-DNA) have been construction. e.g. pGreen.

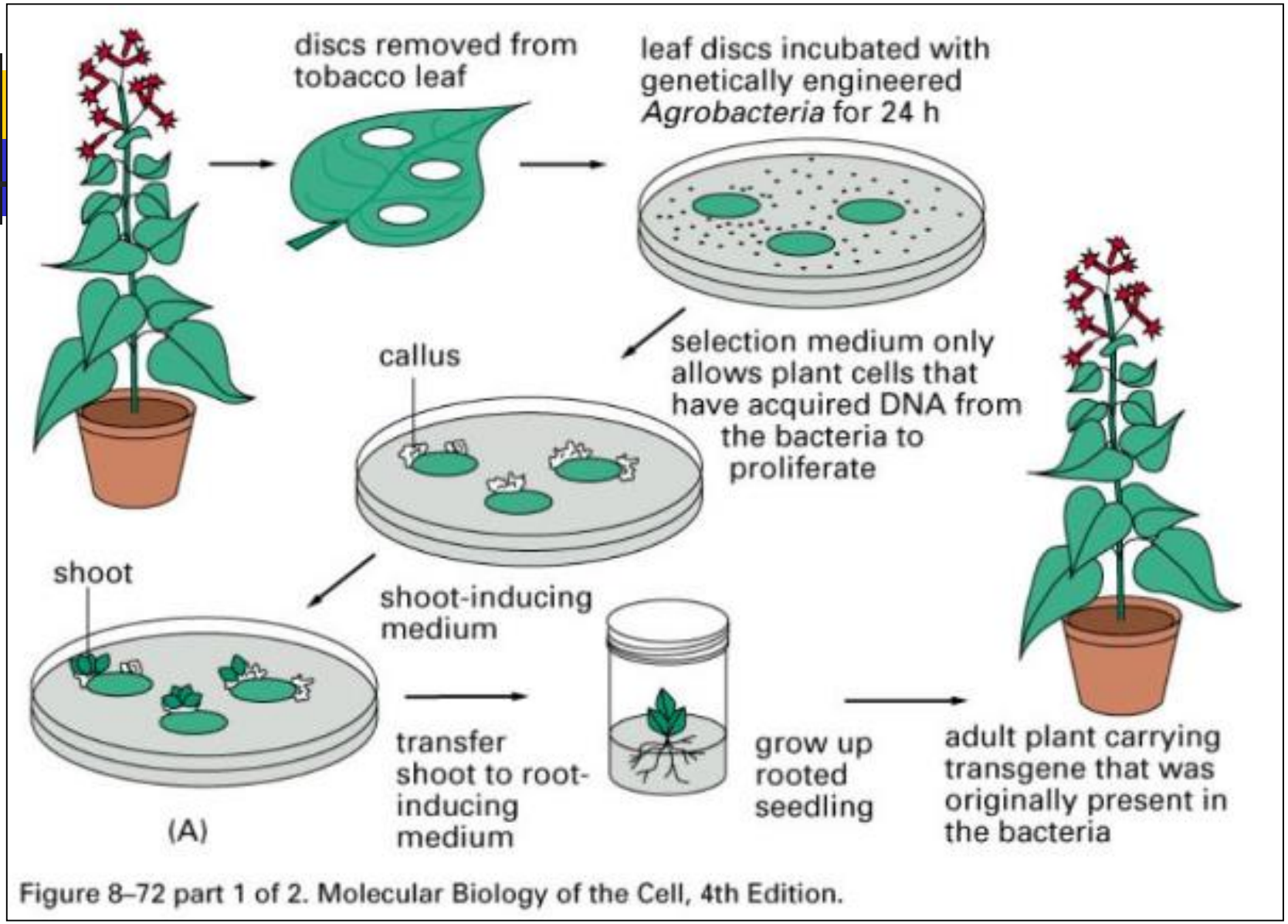


Figure 8–72 part 1 of 2. Molecular Biology of the Cell, 4th Edition.

2. Map-based cloning
Positional cloning

- In the map-based cloning which is also known as positional cloning, one needs to determine the chromosomal location of the gene of interest.
- This includes:
- Genetic localization;
- Marker saturation in the region localized;
- Isolation of large genomic (BAC or YAC) clones;
- Identification of cDNAs and complementation.

cDNA: A duplex DNA sequence complementary to an RNA molecule of interest, carried in a cloning vector.

Map-based cloning
Positional cloning

- Markers linked to the mutated gene;
- Delimiting the region containing the gene of interest;
- The amount of effort required for map-based cloning.

In the fields genetics and evolutionary computation, a locus (plural loci) is the specific location of a gene or DNA sequence on a chromosome. The ordered list of loci known for a particular genome is called a genetic map.
Genetic mapping is the process of determining the locus for a particular biological trait.

Kang,2006

Map-based cloning

Positional cloning

Forward genetics and reverse genetics

- **Forward genetics: From phenotype to gene structure:**

- **Reverse genetics: From gene to phenotype:**

Kang,2006

Map-based cloning

Positional cloning

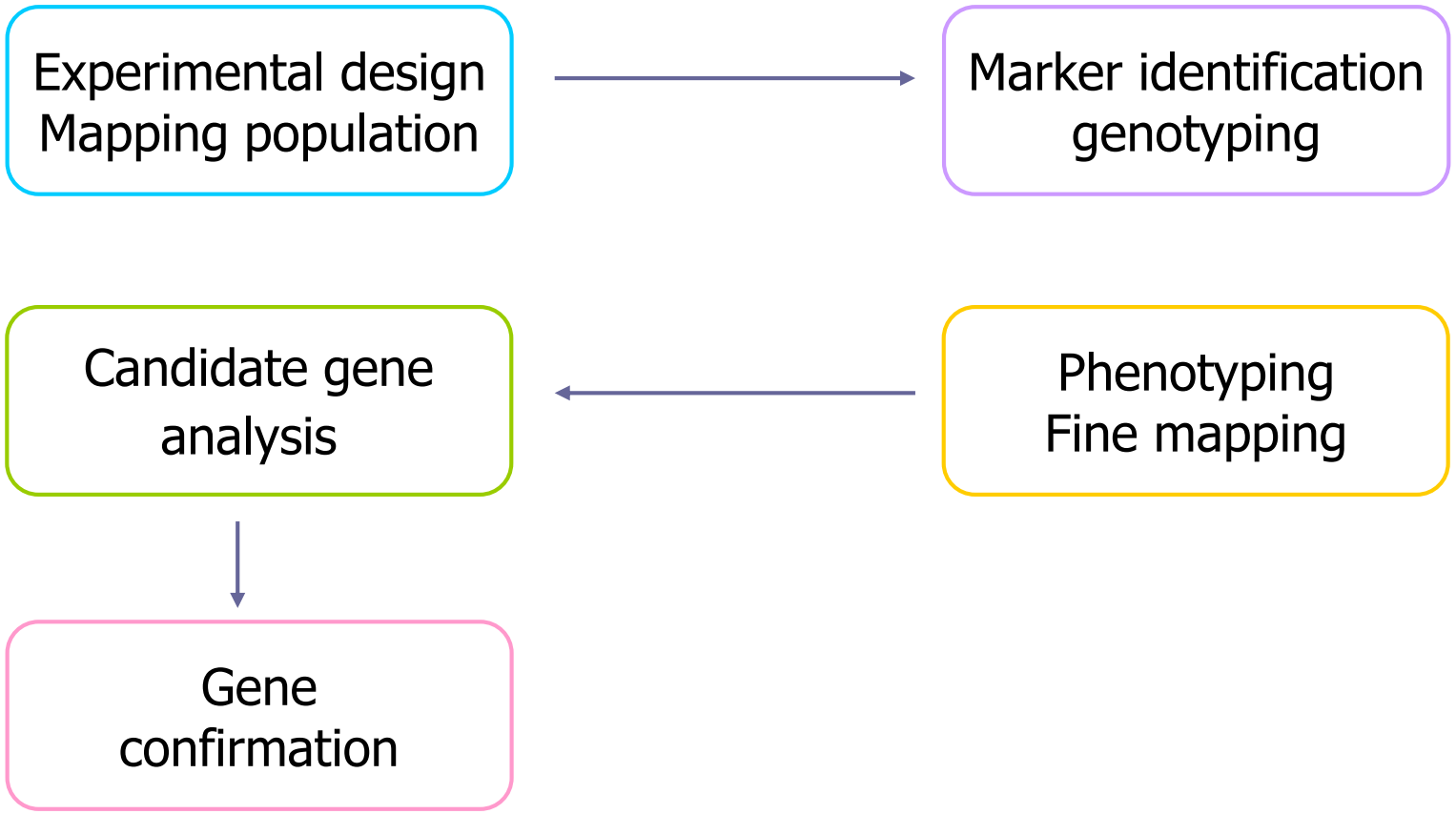
Forward genetics and reverse genetics

- The restriction fragment length polymorphism (RFLP) is the most frequently used method for linking the chromosomal position to a particular trait.
- In this case, co-inheritance of the disease resistance trait with one specific RFLP DNA probe defines the approximate location of the *R* gene on the chromosome.
- The final test can be done after transferring the candidate cDNA fragments into susceptible plants by looking for the acquisition of disease resistance in the transgenic plants.

Map-based cloning

Positional cloning

Forward genetics and reverse genetics



Kang,2006

Map-based cloning

Positional cloning

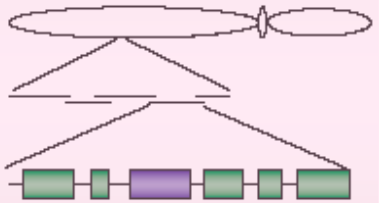
Forward genetics and reverse genetics

Forward genetics

Select a biological process

Generate a random and highly redundant mutant population

Screen a large number of mutagenized M2 plants

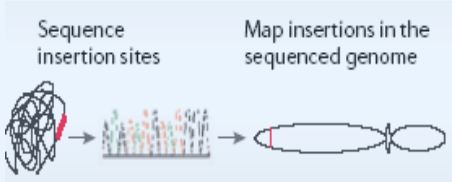


Map and clone the mutation

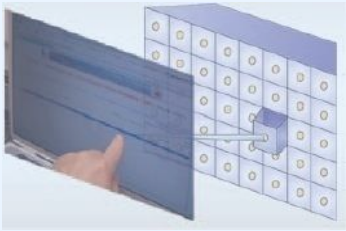
Select a gene or genes

Generate a collection of mutants or tools

Catalogue the mutants in the collection



Select mutants in the gene or genes of interest



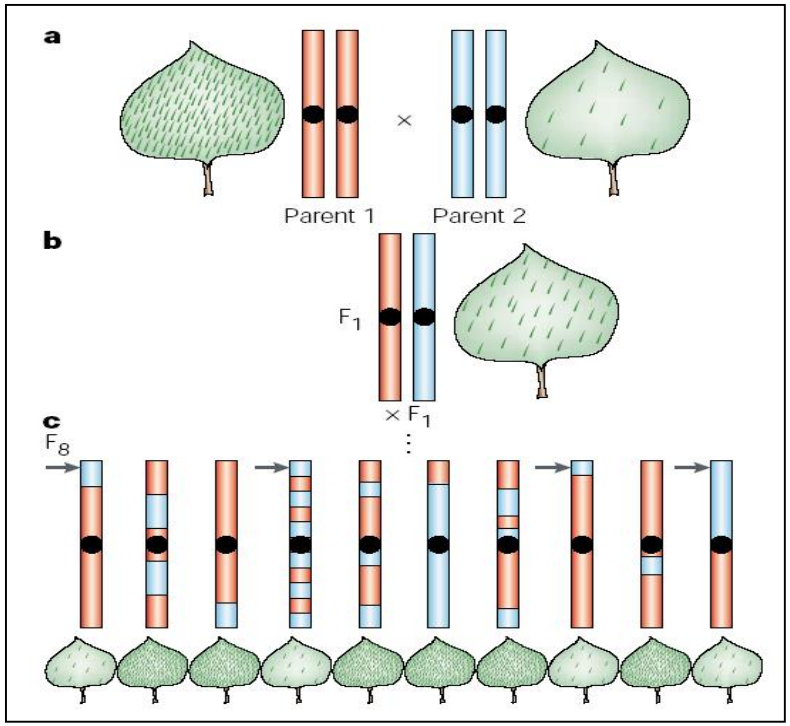
Reverse genetics

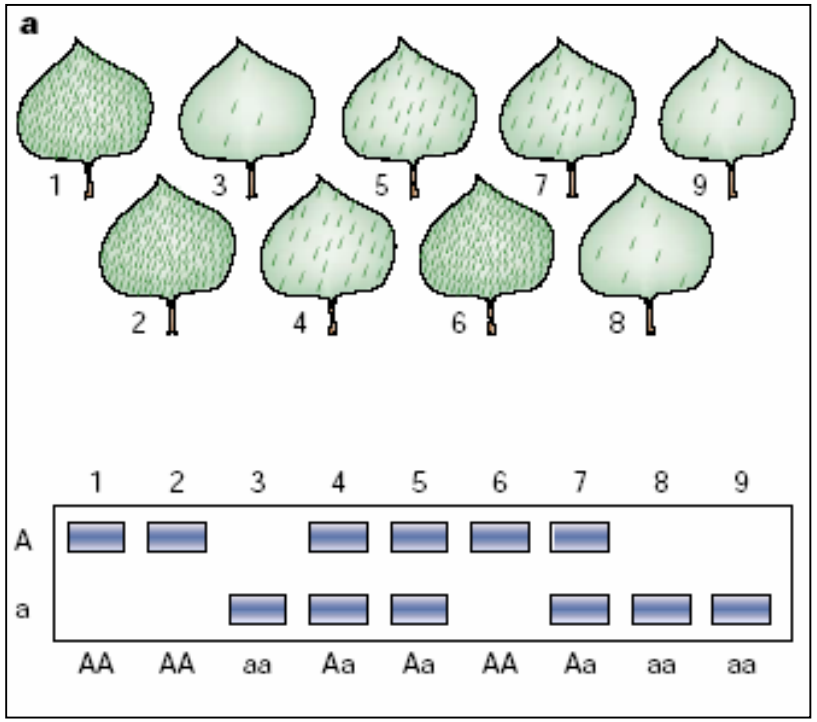
Kang,2006

Map-based cloning

Positional cloning

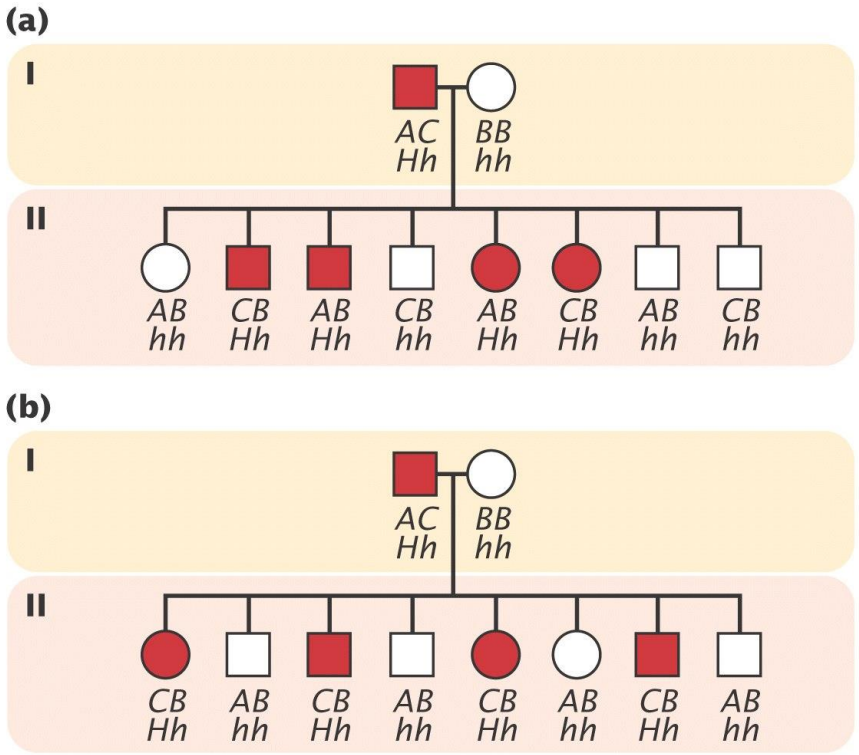
Forward genetics and reverse genetics

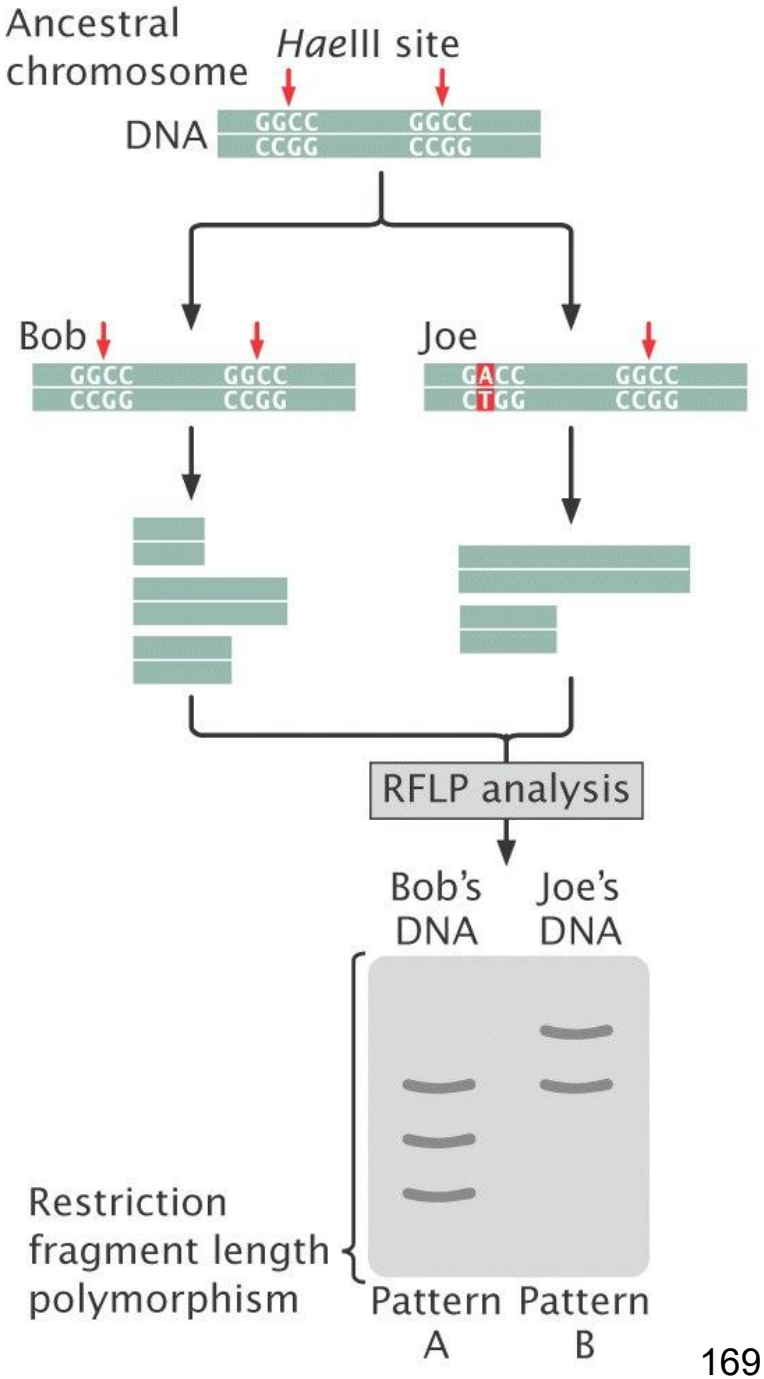




Kang,2006

Restriction Fragment Length Polymorphisms (RFLPs) and Gene Mapping



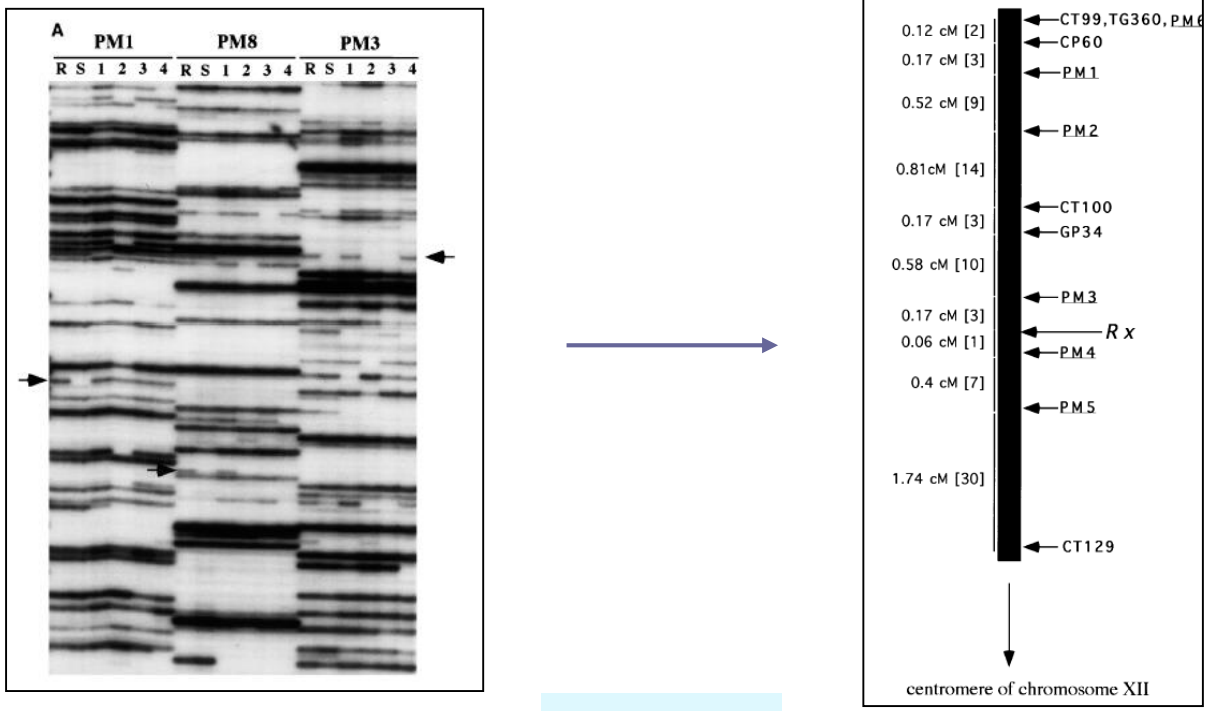


Map-based cloning

Positional cloning

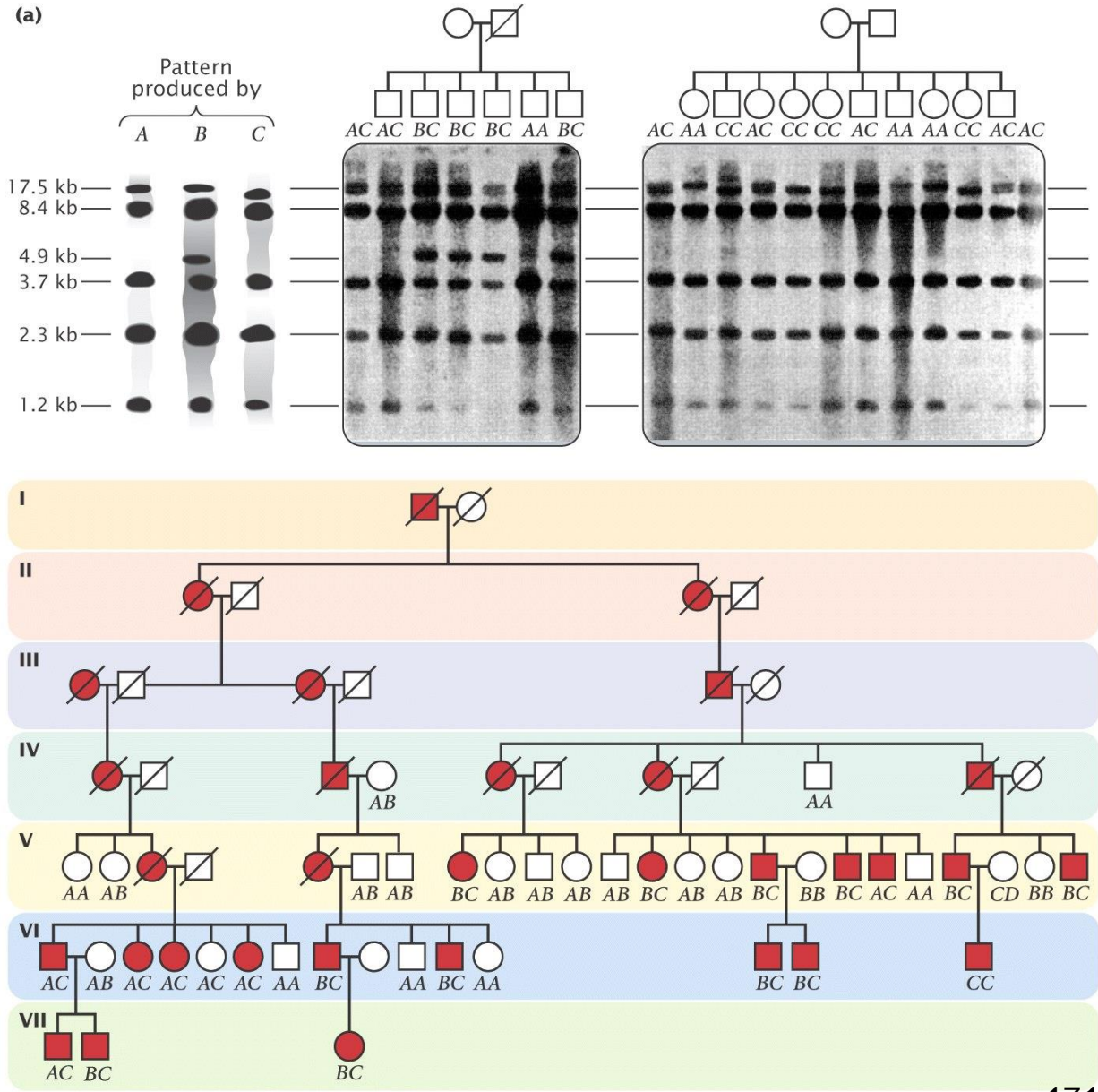
Forward genetics and reverse genetics

- Based on the use of genetic techniques to construct maps showing the position of genes and other sequence features on a genome.



Kang,2006

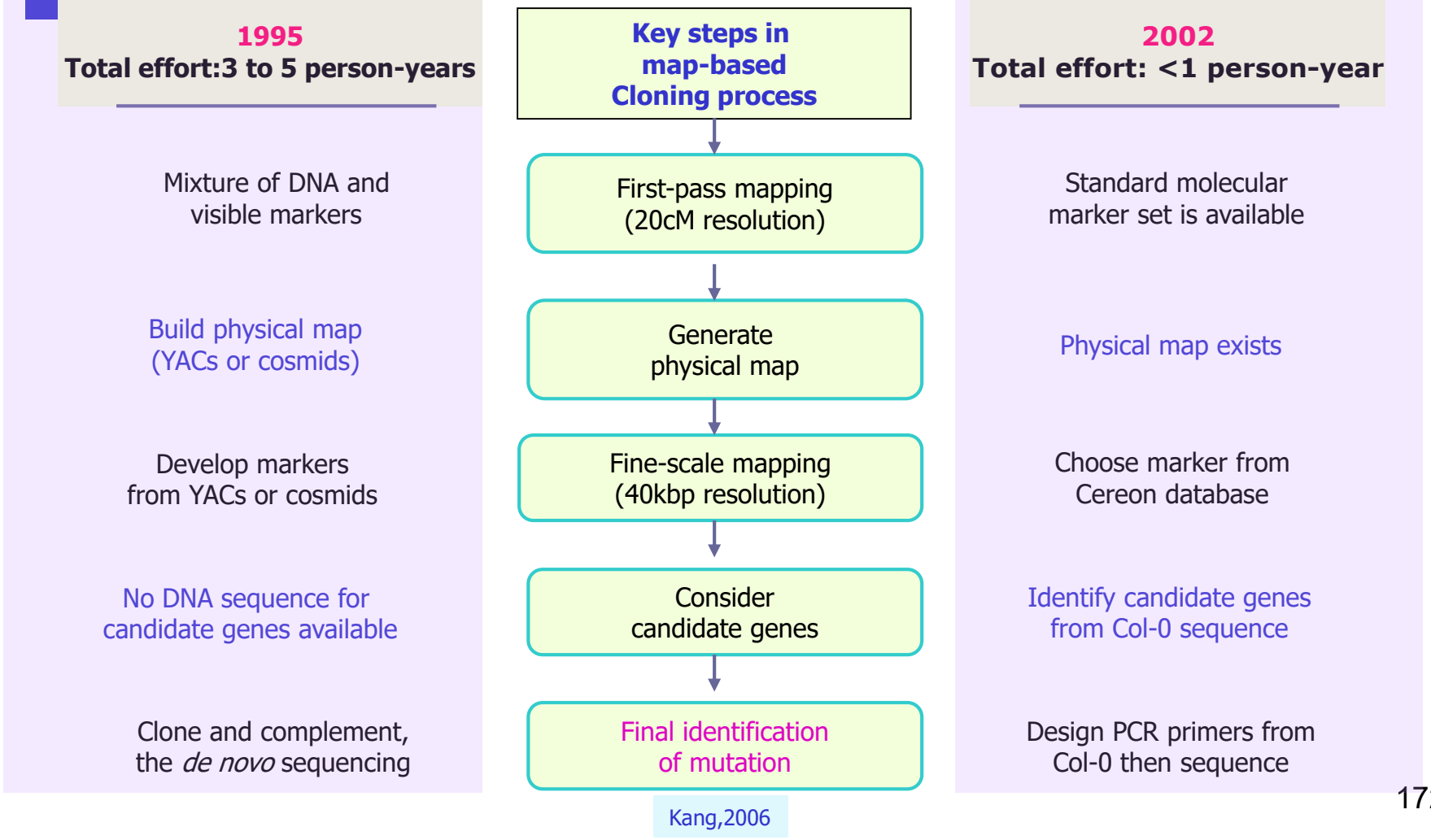
RFLPs and Gene Mapping



Map-based cloning

Positional cloning

Forward genetics and reverse genetics



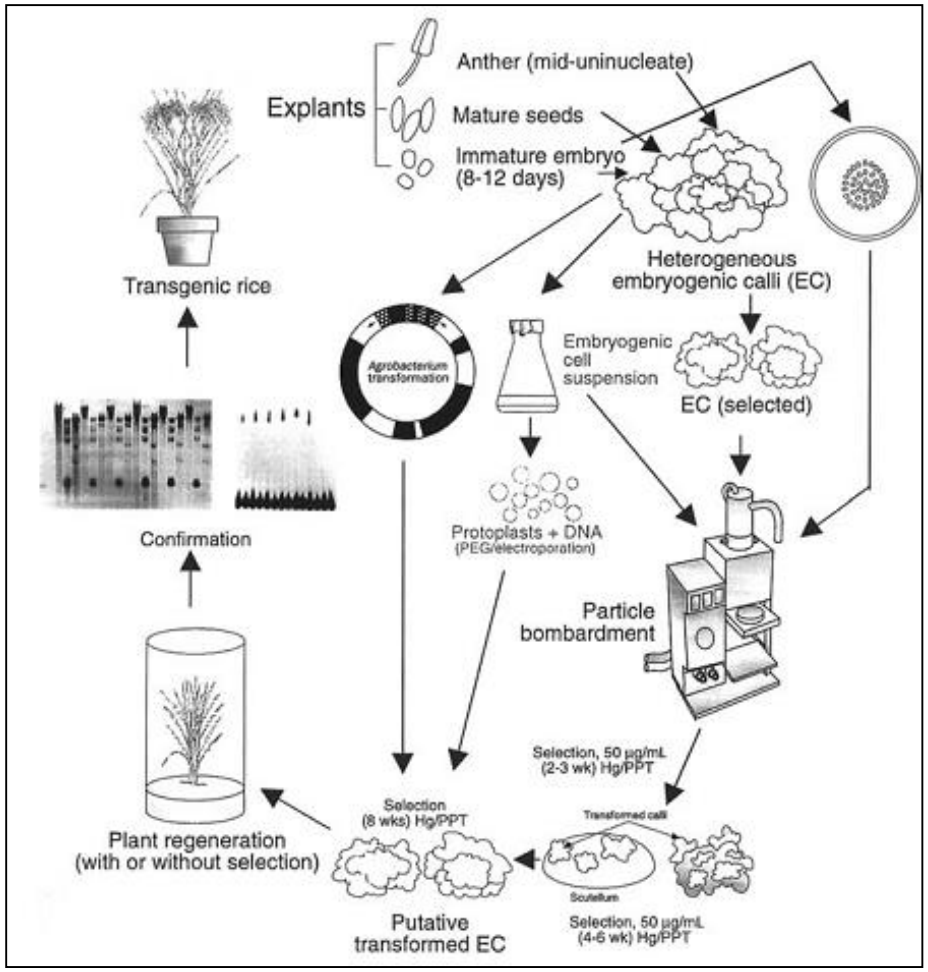
Kang,2006

Other transformation methods

Protoplast techniques

- Another method of plant transformation is to use protoplasts that have been made permeable to foreign DNA by electroporation or polyethylene glycol (PEG) treatment.
- However, a disadvantage of using protoplasts is that it is often difficult to regenerate plants from them as the majority of plant cells are not totipotent.

A schematic protocol for production of transgenic rice plants using biolistic-, protoplast-, and *Agrobacterium tumefaciens* -mediated transformation



Gnanamanickam,2002

Other transformation methods

Biolistic techniques

- Another technique is to use biolistics.
- Here gold particles, coated with DNA, are fired into recipient plants.
- Although expression of foreign DNA is often obtained by this technique, it is usually transient and is frequently quickly lost as the DNA is seldom integrated into the host genome.

Biolistic: This term has been coined from the words “biologic” and “ballistic”; refers to process involving the use of pellets coated with the desired genes that are fired from a gun into seeds or plant tissues in order to get plants expressing these transgenes.

Biolistic technique

- A gene gun used to transform plants by firing gold particles (~1 m diameter) coated with DNA at plant tissue.
- The DNA contains the gene of interest and a selectable marker allowing only transformed cells to proliferate.
- These may then be regenerated into intact plants.

Strange,2003

Genetic Engineering Plants

2. Expression and Function of PR-Protein Genes in Transgenic Plants

R gene or PR-protein genes

Over-expression of PR proteins in transgenic plants

- The *R* genes is specific to a particular host and in some cases to a limited assortment of pathovars.
- The PR proteins on the other hand, would be located in regions where they are most effective against the invading pathogens (for example, the extracellular space).
- Therefore in this defense strategy (Over-expression of PR proteins in transgenic plants), one would ensure that PR proteins would be present in host plants at levels needed for effective resistance (even if the resistance achieved is partial) before the attack by pathogens (mostly fungi) and pests.

Expression and function of PR-protein genes in transgenic plants

- The major plant defense response against plant pathogens involves the biosynthesis and accumulation of pathogenesis-related proteins (PR proteins).
- In some cases, there is a good correlation between a rapid and high-level expression of one or more PR proteins and the resistance reaction of the host plant.
- Overexpression of genes in plants encoding certain PR proteins has been shown to provide protection against specific pathogens.

Datta *et al.*,1999

Expression and function of PR-protein genes in transgenic plants

- These findings have led to the hope that a genetic engineering strategy involving constitutive, high-level expression of combinations of PR proteins with different modes of action against target organisms may provide:
- broad-spectrum, durable resistance to a variety of diseases and pests.

Datta *et al.*,1999

Expression and function of PR-protein genes in transgenic plants

Prospects: Durable resistance using "multigene" combinations

- Breeding for resistance in transgenic plants with multigene combination of PR protein genes (gene pyramiding) may be a good strategy to achieve longer-lasting resistance by reducing the probability of the breakdown of crops' resistance to pathogens, akin to the recent conventional breeding programs that aim to select for more durable resistance using "multigene" combinations.

Genetic Engineering Plant

Resistance gene and defense genes

Xa-21 and PR proteins

- Data are available about a number of transgenic rice plants that have been generated to express major R genes (e.g., *Xa-21* for bacterial blight resistance) and defense genes (chitinases, glucanases, and thaumatin-like proteins) for the management of rice bacterial blight, sheath blight, and other diseases.
- The possibility of pyramiding these genes in transgenic plants to afford higher levels of pathogen suppression presents another good opportunity for biological disease management.

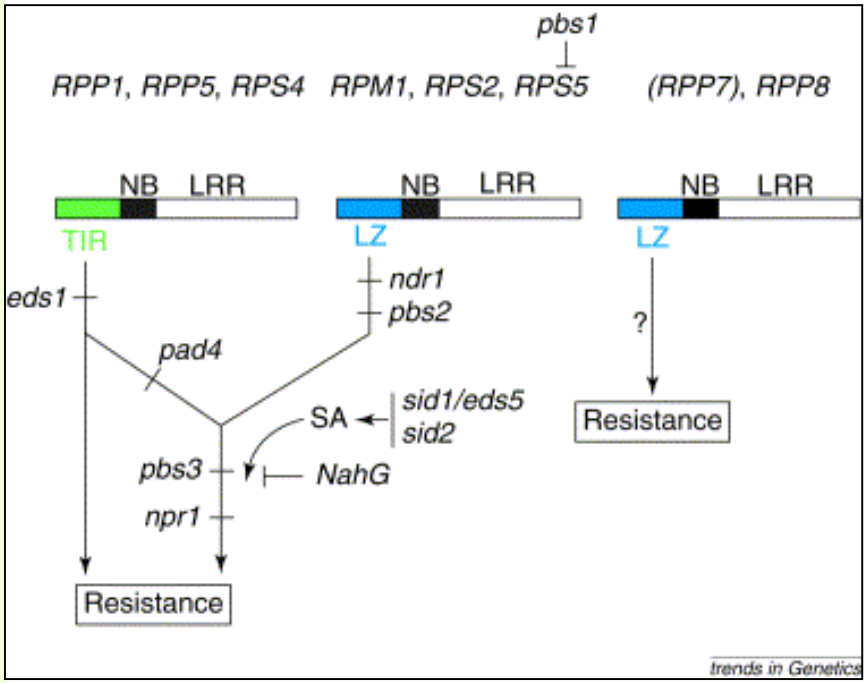
Gnanamanickam,2002

The *R* genes
Overexpression of *NDR*

- It has been reported that overexpression of *NDR1* resulted in:
- Enhanced bacterial disease resistance in *Arabidopsis*, and
- Overexpression of the *Arabidopsis NPR1* gene in Arabidopsis, tomato and rice all led to enhanced broad-spectrum disease resistance.

R gene-mediated signaling pathways in *Arabidopsis*

- Genetic analysis of NB-LRR type *R* genes in *Arabidopsis* has shown that R proteins with an amino-terminal TIR domain predominantly signal through *EDS1*.
- Whereas LZ-containing R proteins require *NDR1* and *PBS2* to initiate defense responses.



Recognized and proposed families of pathogenesis-related proteins

Van Loon and Van Strien,1999

Family	Type member	Properties
PR-1	Tobacco PR-1a	unknown
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	chitinase type I, II, IV, V, VI, VII
PR-4	Tobacco "R"	chitinase type I, II
PR-5	Tobacco S	thaumatin-like
PR-6	Tomato Inhibitor I	proteinase-inhibitor
PR-7	Tomato P_{6g}	endoproteinase
PR-8	Cucumber chitinase	chitinase type III
PR-9	Tobacco "lignin-forming peroxidase"	peroxidase
PR-10	Parsley "PR1"	"ribonuclease-like"
PR-11	Tobacco class V chitinase	chitinase type I
PR-12	Radish Rs-AFP3	defensin
PR-13	*Arabidopsis* THI2.1	thionin
PR-14	Barley LTP4	lipid-transfer protein

Edreva,2005

Recognized and proposed families of pathogenesis-related proteins

Family	Type member	Properties	Gene symbols
PR-1	Tobacco PR-1a	Unknown	*Ypr1*
PR-2	Tobacco PR-2	β-1,3-glucanase	*Ypr2*, [*Gns2* (‘*Glb*’)]
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII	*Ypr3*, *Chia*
PR-4	Tobacco ‘R’	Chitinase type I, II	*Ypr4*, *Chid*
PR-5	Tobacco S	Thaumatin-like	*Ypr5*
PR-6	Tomato Inhibitor I	Proteinase-inhibitor	*Ypr6*, *Pis* (‘*Pin*’)
PR-7	Tomato P_{69}	Endoproteinase	*Ypr7*
PR-8	Cucumber chitinase	Chitinase type III	*Ypr8*, *Chib*
PR-9	Tobacco “lignin-forming peroxidase”	Peroxidase	*Ypr9*, *Prx*
PR-10	Parsley “PR1”	Ribonuclease-like	*Ypr10*
PR-11	Tobacco “class V” chitinase	Chitinase, type I	*Ypr11*, *Chic*
PR-12	Radish Rs-AFP3	Defensin	*Ypr12*
PR-13	Arabidopsis THI2.1	Thionin	*Ypr13*, *Thi*
PR-14	Barley LTP4	Lipid-transfer protein	*Ypr14*, *Ltp*
PR-15	Barley OxOa (germin)	Oxalate oxidase	*Ypr15*
PR-16	Barley OxOLP	Oxalate-oxidase-like	*Ypr16*
PR-17	Tobacco PRp27	Unknown	*Ypr17*

More details at http://www.bio.uu.nl/~fytopath/PR-families.htm.

Van Loon *et al.*,2006

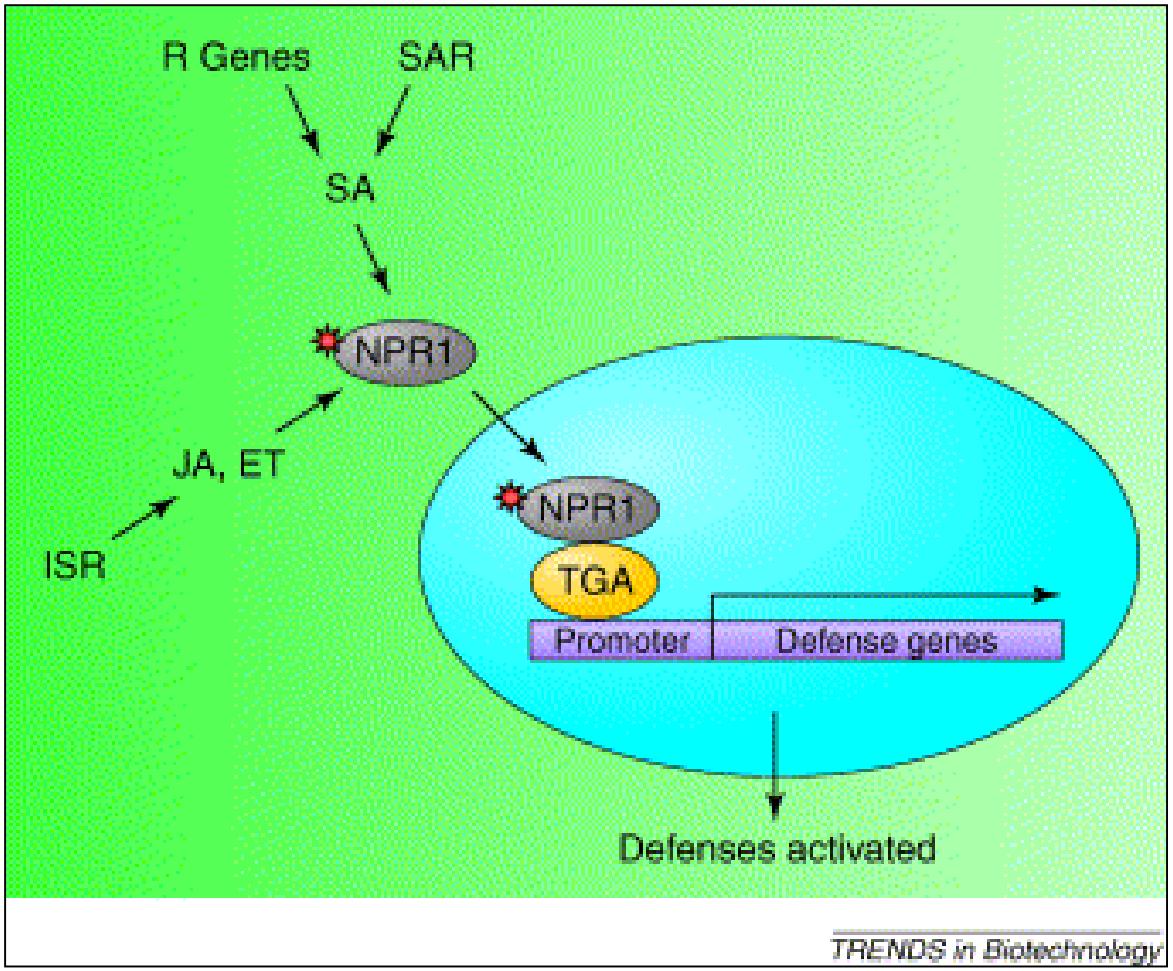
Mechanism of actions

Transformation with constructs causing the overexpression of essential regulatory genes in systemic acquired resistance

- *NPR1* (non-expresser of PR genes) also known as NIM1 (for non-immunity) or *SAI1* (for salicylic acid-insensitive) plays a crucial role in local and systemic acquired resistance (SAR) in the model plant, *Arabidopsis thaliana*.

1. Mutations in the gene result in loss of the ability to accumulate pathogenesis-related proteins (PRs) and susceptibility to fungal and bacterial pathogens even if the plants are pre-treated with inducers of SAR.
2. Conversely, some plants transformed with the *NPR1* gene under the control of the CaMV35S promoter express two- to three-fold higher levels of NPR1 protein but no increase in the expression of PR genes.

Regulation of defense gene expression by NPR1



Mechanism of actions

Correlation of *NPR1* and *PR* genes activities

- Although over-expression of *NPR1* is required for enhanced resistance, PR proteins were not expressed until the plant was challenged by a pathogen.
- In case of fungi, the transgenics overexpressing *NPR1* were more resistant to *Peronospora parasitica* if the greater expression of the *PR* genes *PR1, PR2* and *PR5* were occured.

Genetic engineering with PR-protein genes

Against fungal diseases

- Among the PR-protein genes, chitinase and *β*-1,3-glucanase genes were most attractive because of their strong *in vitro* antifungal activities.
- These two enzymes hydrolyze chitin and β-1-3-glucans which are structural components of the cell walls of several fungi.
- Transgenic tobacco and canola plants overexpressing a bean chitinase gene were found to be more resistant to *Rhizoctonia solani* infection as shown by the delayed development of disease symptoms (Broglie *et al.*,1991).
- Studies with transgenic tobacco plants expressing a soybean *β* -1,3-glucanase have improved resistance to:
- *Alternaria alternata*,
- *Phytophthora parasitica*, and
- *Peronospora tabacina.*

Transgenic plants
PR-Proteins as antifungal proteins

- The constitutive over expression of tobacco class I PR-2 and PR-3 transgenes in potato plants enhanced their resistance to *Phytophthora infestans*, the causal agent of late blight.
- Transgenic cucumber harboring the rice chitinase genes exhibited enhanced resistance against gray mold, *Botrytis cinerea*.
- Constitutive expression of the *PR-1* gene has no apparent effect on symptoms development in transgenic tobacco inoculated with tobacco mosaic virus.

Transgenic plants

Bacterial chitinase as antifungal proteins

- At least 50% of the chitinase protein produced in transgenic tobacco cells with the *Serratia marcescens* chitinase.
- Introduction of bacterial chitinase gene from *S. marcescens* in transgenic tobacco cells showed up to an eightfold increase in the amount of chitinase protein in the plants and conferred resistance to *R. solani*.

Transgenic plants

Bacterial chitinase as antifungal proteins

- Expression of a PR-protein gene in rice was successive.
- Several independent transgenic lines were regenerated, with chitinase expression ranging from 2- to 19-fold over controls.
- The number and size of lesions in transgenic plants were smaller compared to control.
- The degree of resistance displayed by the transgenic plants to the *R. solani* correlated with the level of chitinase expression.

Transgenic plants

Bacterial chitinase as antifungal proteins

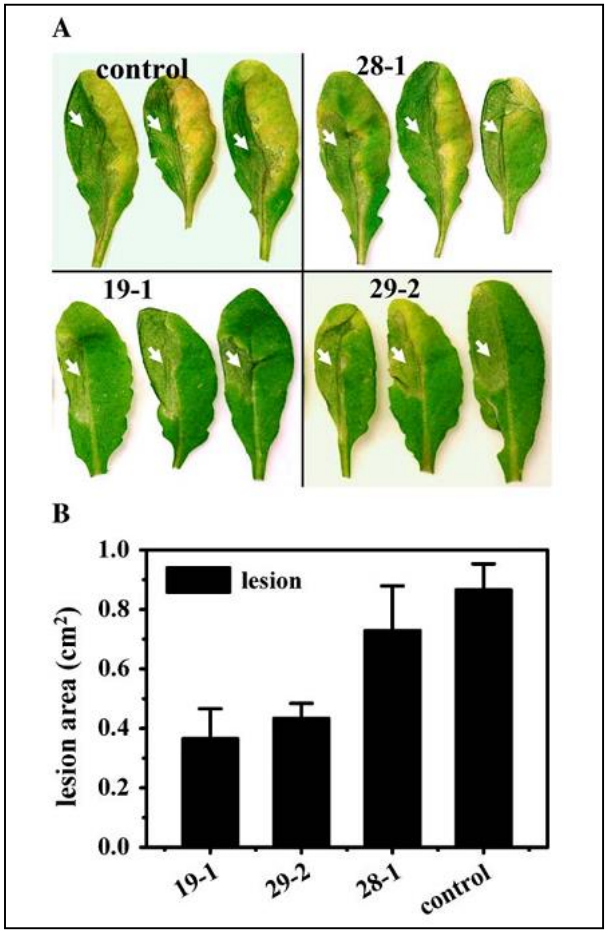
Transgenic plant	Gene	Source	Pathogen
Broccoli	Endochitinase	Trichoderma harzianum	*Alternaria sp.*
Cucumber	Chitinase (RCC2)	Oryza sativa	Botrytis cinerea
Elite indica rice	PR-3 chitinase (RC7)	*O. sativa*	R. solani
Grapevine	Class-I chitinase (RCC2)	*O. sativa*	Uncinula necator
Indica rice	Classs-I chitinase (Chi11)	*O. sativa*	R. solani
Japonica rice	Class-I chitinase (Cht-2, Cht-3)	*O. sativa*	*Magnaporthe grisea*
Potato	Endochitinase [ThEn-42 (chit42)]	*T. harzianum*	Alternaria alternata, A. solani, Botrytis cinerea, R. solani
Strawberry	Chitinase	*O. sativa*	*Sphaerotheca humuli*
Tobacco	Endochitinase [ThEn-42 (chit42)]	*T. harzianum*	*Alternaria alternata, A. solani, Botrytis cinerea, R. solani*
Wheat	Chitinase (chi11)	*O. sativa*	*Fusarium graminearum*

Gohel *et al.*,2006

Transgenic plants

Antibacterial activity of transgenic Arabidopsis plants against *Pseudomonas syringae* pv. *tomato* DC3000

- (A) Severity of disease symptoms among control (transformed with vector pCAMBIA302 only), ZmPR10 transgenic line 28-1 and ZmPR10.1 transgenic lines (19-1 and 29-2) 4 d after inoculation.
- The arrows indicate the areas of inoculation.
- (B) Lesion size of filtrated transgenic Arabidopsis leaves. Means are shown with SE calculated from 12 infiltrated leaves from three plants.



Rong-Xie *et al.*,2010

Genetic Engineering Plants

3. Genes silencing

Gene silencing
Turning off gene

- Gene silencing is a general term describing epigenetic processes of gene regulation.
- The term gene silencing is generally used to describe the "switching off" of a gene by a mechanism other than genetic modification.
- That is, a gene which would be expressed (turned on) under normal circumstances is switched off by machinery in the cell.

Gene silencing
Turning off gene

- Genes are regulated at either the transcriptional or post- transcriptional level:
- Transcriptional gene silencing is the result of histone modifications makes it inaccessible to transcriptional machinery (RNA polymerase, transcription factors, etc.).
- Post-transcriptional gene silencing is the result of mRNA of a particular gene being destroyed.
- The destruction of the mRNA prevents translation to form an active gene product (in most cases, a protein).

Gene silencing
Turning off gene

- A common mechanism of post-transcriptional gene silencing is RNAi.
- Mechanisms of gene silencing also protect the organism's genome from transposons and viruses.
- Gene silencing thus may be part of an ancient immune system protecting from such infectious DNA elements.

Gene silencing

Candidate genes for plant transformation in order to enhance resistance

- Introduction of additional copies of genes in order to increase their expression often, paradoxically, results in decreased expression.
- One promising application of this technology is in the control of crown gall.
- Here it is proposed that transformation of plants with the genes that the pathogen uses for the production of IAA and cytokinins would give durable resistance since these genes are responsible for the unorganized growth of tumours in infected plants.

Control of crown gall by genes silencing

Transgenes with bacterial sequences suppress bacterial replication

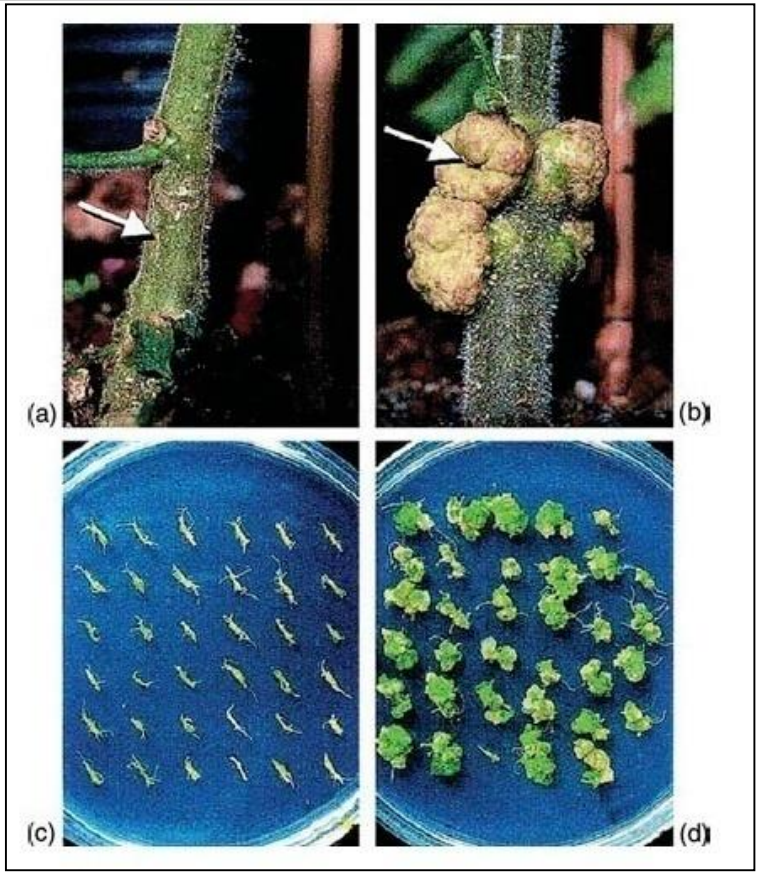
- Control of crown gall by silencing of the genes used by the bacterium for the synthesis of IAA and cytokinins:

a) tomato transgene;

b) tomato control;

c) *Arabidopsis* transgene;

d) *Arabidopsis* control.



Strange,2003

Genetic Engineering Plants

4. Enhancing levels of reactive oxygen species

Enhancing levels of reactive oxygen species

- Wu *et al.*,1995 transformed potatoes with an H_2O_2-generating glucose oxidase gene from *Aspergillus niger*.
- Transgenic tubers were highly resistant to soft rot caused by *Erwinia carotovora* but the resistance was abolished by catalase confirming that H_2O_2 was responsible.
- Transgenic plants were also resistant to *Phytophthora infestans* and *Verticillium dahliae*.

Genetic Engineering Plants

5. With genes encoding enzymes which degrade toxins produced by pathogens

Albicidins

Toxin and antibiotics

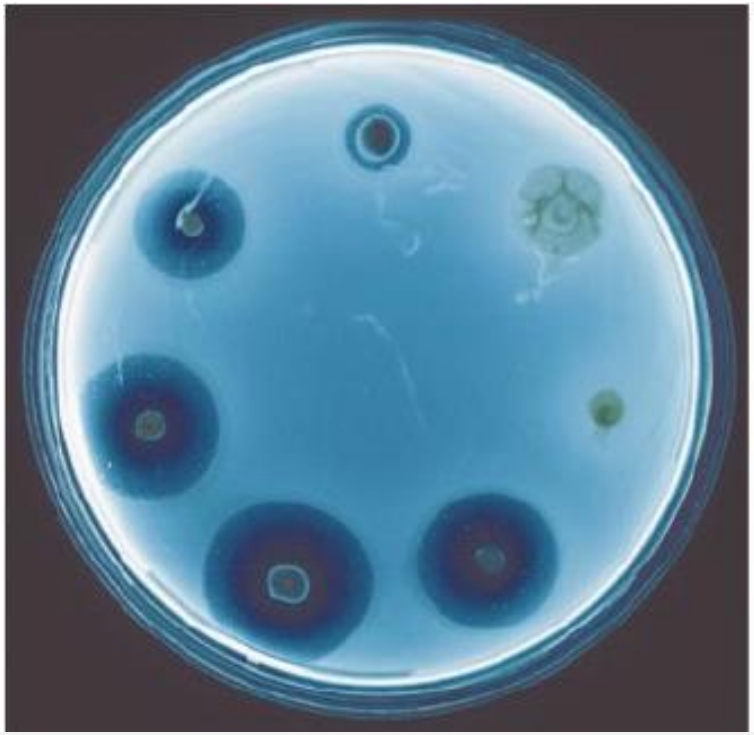
- Albicidins such as Abscisic acid (ABA) are a family of phytotoxins and antibiotics produced by the xylem invading bacterium *Xanthomonas albilineans*.
- Symptoms of leaf scald include chlorosis, necrosis, wilting and sometimes sudden death of the plant after a long latent period.
- The chlorotic symptoms are caused by inhibition of DNA synthesis in chloroplasts caused by the toxin.
- Tox^- mutants are unable to cause disease symptoms.

Sugarcane leaf scald disease
X. albilineans

- Acute leaf scald symptoms, including wilting, in-arching and death of leaves in sugarcane.

Albicidins as antibiotics

- Inhibition of *E. coli* lawn growth around colonies of *X. albilineans* due to albicidin antibiotics.
- Other tested *Xanthomonas* spp. (top right) are not inhibitory.



Chlorosis-inducing isolates of *X. albilineans* produce a family of antibiotics called albicidins in culture.

Birch,2001

Antipathogenic approach

New control strategies

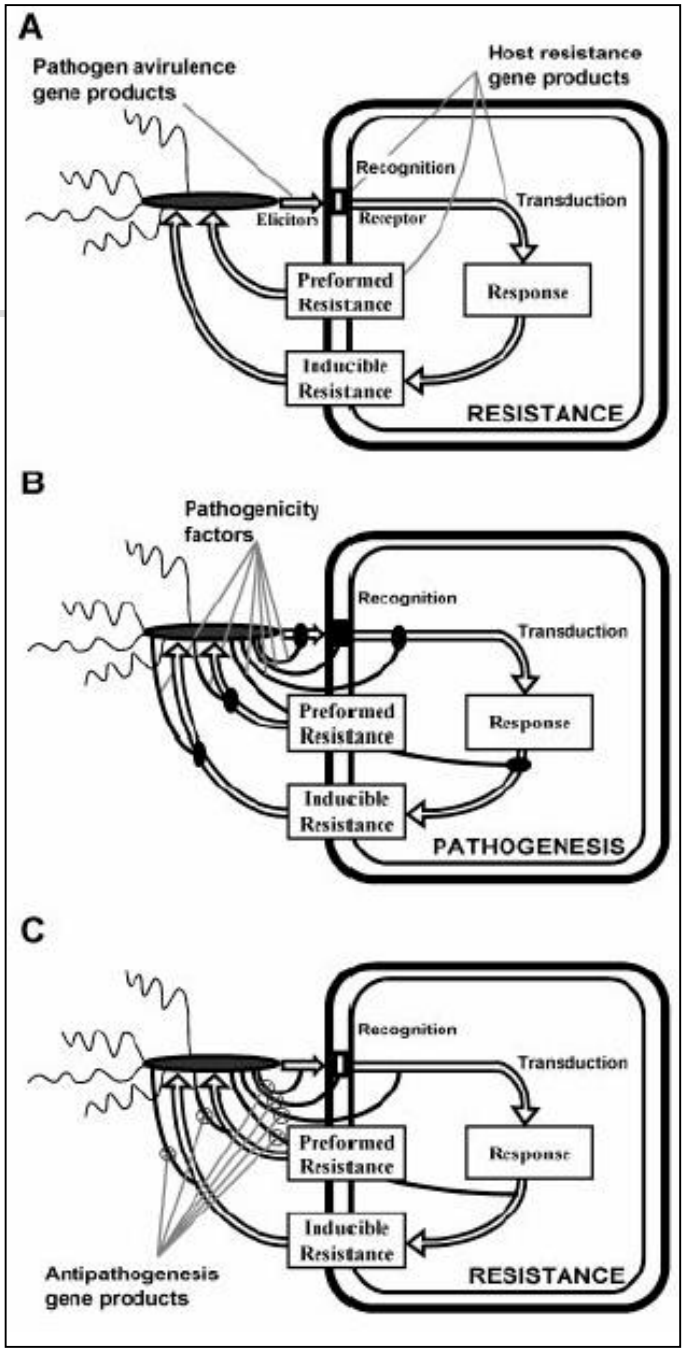
- The antipathogenesis approach to disease control involves the identification of weaknesses in a pathogenesis strategy, as targets for the development of effective disease control measures.
- e.g.
- Investigation of the molecular basis for the enigmatic patterns of symptom development (such as albicidins as key pathogenicity factors in sugarcane leaf scald led to some unexpected outcomes, including:

1. The discovery of a new class of promising antibiotics,
2. The novel resistance genes to rescue susceptible varieties.

Birch,2001

Antipathogenic approach
New control strategies

- Plant defences (A) are effective against most microbes, but may be breached by pathogenicity factors (B).
- The antipathogenesis approach aims to understand, then interfere with, key pathogenicity factors.
- For example, by the use of novel genes for inactivation of pathogenicity factors (C).



Birch,2001

Albicidins resistance

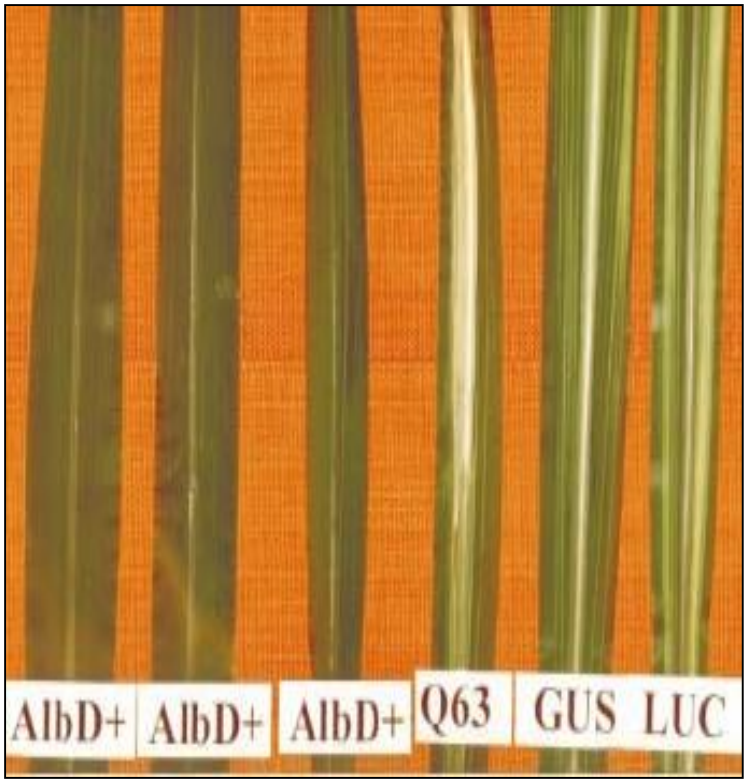
- Several genes that confer resistance to albicidins have been cloned from heterologous, biocontrolling bacteria, such as *Klebsiella oxytoca* (*albA*), *Alcaligenes denitrificans* (*albB*), *Pantoea dispersa* (syn. *Erwinia herbicola*) (*albD*), and from *X. albilineans* itself (*albD*).
- These genes may be useful candidates for transfer into the sugarcane genome.
- Indeed, expression of *albD* in transgenic sugarcane resulted in reduced chlorotic disease symptoms and conferred resistance to systemic multiplication of the pathogen.

Transformation with genes encoding enzymes which degrade toxins produced by pathogens

- Zhang, Xu and Birch (1999) generated transgenic sugarcane plants that expressed a gene from *Pantoea dispersa* which detoxified albicidin, a potent toxin from *Xanthomonas albilineans*.
- Plants expressing the gene at levels of 1–10 ng of enzyme per mg of leaf protein did not develop chlorotic disease symptoms in inoculated leaves, whereas all untransformed control plants developed severe symptoms.
- As mentioned earlier, transgenic lines with high activity of the enzyme in young stems were also protected against systemic multiplication of the pathogen.

Transformation with genes encoding enzymes which degrade toxins produced by pathogens

- Transgenic sugarcane plants expressing *albD* do not develop chlorotic disease symptoms in inoculated leaves under conditions where all untransformed control plants develop severe symptoms.
- Q63 is parent variety which is untransformed or transformed with GUS or LUC reporter genes.



Genetic Engineering Plants

6. Modifying the phytoalexin response

Modifying the phytoalexin response

- Some consideration has been given to introducing genes into plants that would allow them to synthesize 'foreign' phytoalexins.
- This would appear to be a considerable technical challenge since the metabolic pathways leading to most phytoalexins is long.
- One possibility would be to introduce genes which encode enzymes that alter the last few steps in the biosynthetic sequence.
- Hain and co-workers (1993) introduced a gene encoding stilbene synthase from grape-vine into tobacco, a plant that normally produces terpenoid phytoalexins.
- On challenge, the transgenic plant synthesized the stilbene, resveratrol and was more resistant to attack by *Botrytis cinerea*.

Genetic Engineering Plants

7. Antibacterial peptides (AMPs)- mediated resistance

Transformation with genes encoding antimicrobial peptides

- Antimicrobial peptides or proteins are natural peptides alternative to chemical antibiotics and a potential for applied biotechnology.
- A large group of low molecular weight natural compounds that exhibit antimicrobial activity has been isolated from animals and plants during the past two decades.
- A resulting new generation of anti microbial peptides (AMPs) with higher specific activity and wider microbe-range of action could be constructed, and hopefully endogenously expressed in genetically-modified organisms.

Transformation with genes encoding antimicrobial peptides

- More recently, a number of genes encoding some of the constitutive defence peptides against pathogens, have been used to transform plants and their expression has been correlated with enhanced resistance.
- Several other such genes but from non-plant sources such as insects and mammals have also been used.
- The spectrum of activity of individual peptides varies but, as a whole, those with activity against all the major classes of plant pathogens, viruses, bacteria, fungi and nematodes, can be found.

Anti-microbial peptides (AMP)
Natural peptides

- Antimicrobial peptides (AMPs) with α-helical structures are ubiquitous and found in many organisms.
- They are a common component of innate defense mechanisms in the animal kingdom and help to control normal microbial flora and combat pathogens (Tossi *et al.*,2000).

Anti-microbial peptides (AMP)

Natural peptides

- Anti-microbial peptides (AMP) make up a part of the innate immunity of most organisms and are often involved in the immune system's first line of defense when faced with an invader.
- AMPs can be found in:

1. Prokaryotic
2. Eukaryotic
3. Vertebrates
4. Invertebrates (Reddy,2004).

- Antimicrobial peptides are so widespread that they are likely to play an important protective role.

Plant peptides

- It is estimated that there are 250,000 to 500,000 species of plants on Earth.
- A relatively small percentage (1 to 10%) of these are used as foods by both humans and other animal species.
- It is possible that even more are used for medicinal purposes.

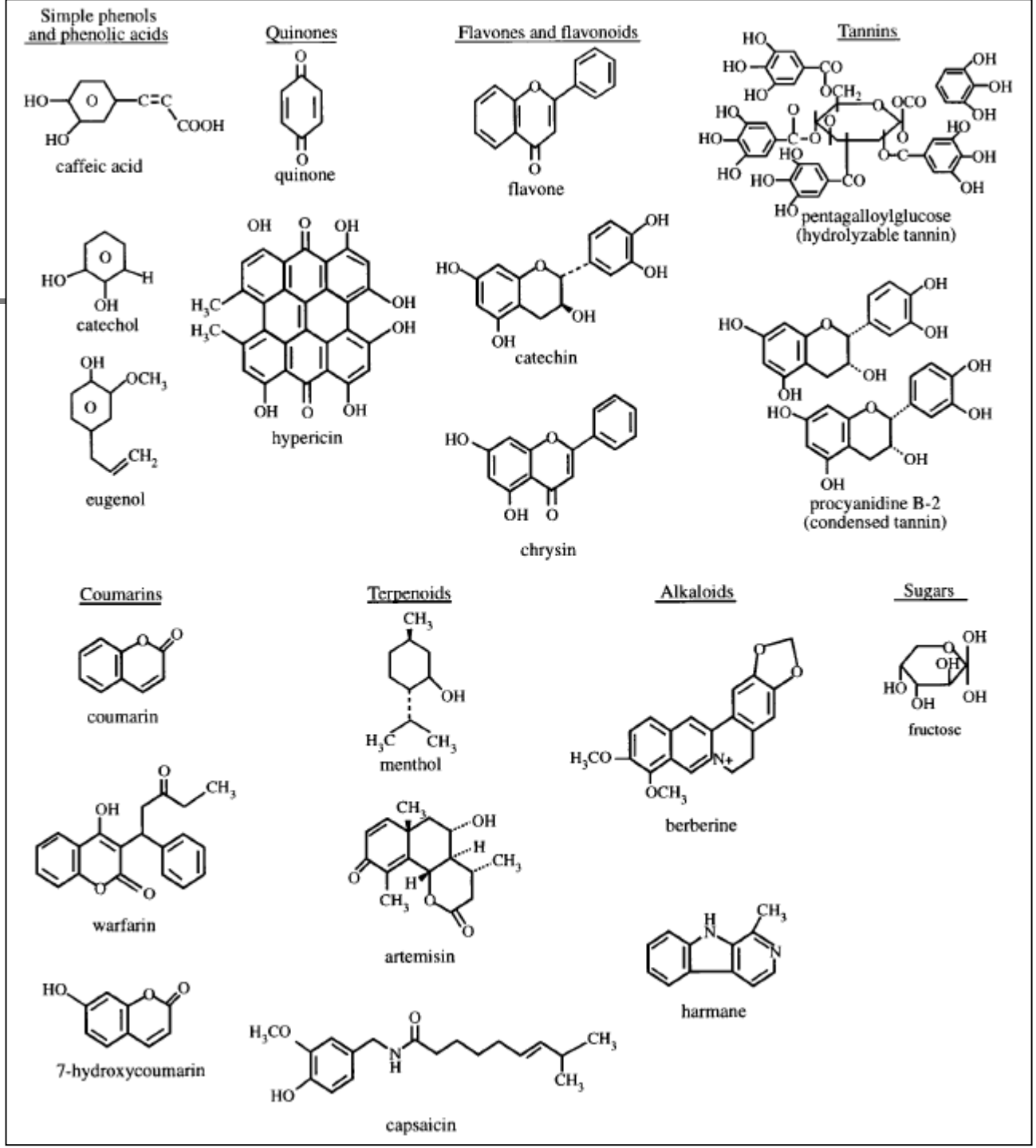
Anti-microbial peptides (AMPs)

- Most AMPs are small (typically less than 10 kDa), are cationic (positively charged due to an abundance of arginine and lysine amino acids throughout the peptide), hydrophobic, and exhibit a broad range of activity.
- Because the cell membranes of many microorganisms are anionic (negatively charged) and eukaryotic cell membranes are electrically neutral, the positively charged AMP automatically has an affinity for the negatively charged microbial membrane.

Major groups of antimicrobial compounds from plants

- Phenolics and Polyphenols;
- Quinones- Aromatic rings with two ketone substitutions;
- Flavones, flavonoids, and flavonols;
- Tannins- A group of polymeric phenolic substances;
- Coumarins - Phenolic substances made of fused benzene and a pyrone rings);
- Alkaloids;
- Terpenoids and Essential Oils;
- Lectins and Polypeptides e.g. Thionins.

Structures of common antimicrobial plant chemicals



Cowan,1999

Antimicrobial secondary metabolites
Mode of action of phytochemicals

- Plants have limitless ability to synthesize aromatic secondary metabolites, most of which are phenols or their oxygen-substituted derivatives.
- Important subclasses in this group of compounds include: phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins.
- These groups of compounds show antimicrobial effect and serves as plant defense mechanisms against pathogenic microorganisms.

Class	Sub-class	Mechanism
Phenolics	Simple phenols	Membrane disruption, substrate deprivation
Phenolic acids	Phenolic acids	Bind to adhesins, complex with cell wall, inactivate enzymes
Terpenoids, essential oils		Membrane disruption
Alkaloids		Intercalate into cell wall
Tannins		Bind to proteins, enzyme inhibition, substrate deprivation
Flavonoids		Bind to adhesins, complex with cell wall, Inactivate enzymes
Coumarins		Interaction with eucaryotic DNA
Lectins and polypeptides		Form disulfide bridges

Gurjar *et al.*,2012

Botanicals produced by plants having antimicrobial activity

Common name	Scientific name	Compound	Class	Activity
Apple	*Malus pumila* Mill.	Phloretin	Flavonoid derivative	General
Ashwagandha	*Withania somnifera* Dunal.	Withafarin A	Lactone	Bacteria, fungi
Bael tree	*Aegle marmelos* Linn.	Essential oil	Terpenoid	Fungi
Blue gum tree	*Eucalyptus globulus* Labill.	Tannin	Polyphenol	Fungi, Bacteria, Viruses
Onion	*Allium cepa* Linn.	Allicin	Sulfoxide	Fungi, Bacteria
Thyme	*Thymus vulgaris* Linn	Caffeic acid	Terpenoid	Fungi, Bacteria, viruses
Turmeric	*Curcuma longa* Linn.	Curcumin	Terpenoids	Fungi, Bacteria, protozoa
Thorn apple	*Datura stramonium* Linn.	Hyoscymine Scopolamine	Alkaloids	Fungi
Black pepper	*Piper nigrum* Linn.	Piperine	Alkaloid	Fungi
Castorbean	*Ricinus communis* Linn.	Ricinine Ricininoleic	Alkaloids	Fungi
Neem/Margosa tree	*Azadirachta indica* A.Juss.	Azadirachtin	Terpenoides	Fungi, Bacteria
Garlic	*Allium sativum* Linn.	Allicin	Solfoxide	Fungi, Bacteria

Gurjar *et al.*,2012

Botanicals produced by plants having antimicrobial activity

Plant	Part used	Preparations	Diseases/pathogen
Datura/thorn apple (*D.stamonium*) *Calotropis procera* (Ait.) R. Br. *Oscimum* spp.	Root, stem, Leaf, flowers	Crude extract	*Curvularia lunata*
Turmeric (*Curcuma longa* Linn.), Ginger (*Zingiber officinale* Rosc.)	Rhizome	Crude extract	*Phytopthora infestans*, *Fusarium solani*, *Pyricularia oryzae*
Perslane (*Portulaca olaracea* Linn.)	Leaf	Crude extract	*Helminthosporium maydis*
Hena (*Lowsonia inermis* Linn.)	Leaf	Crude extract	*Dreshslera oryzae*
Neem/Margosa (*Azadirachta indica* A.Juss.), Sugar apple (*Annona squamosa* Linn.), Holy basil (*Oscimum sanctum* Linn.)	Leaf, Stem Bark, root	Crude extract	Anthracnose of pepper
Neem/Margosa (*Azadirachta indica* A.Juss.),	Seed kernel	Oil	*A. alternata*
Ambrosioides Linn., *Oscimum* spp.	Leaf	Essential oils	*Aspergillus flavus*
Garlic (*Allium sativum* Linn.), Datura (*D. stramonium* Linn.)	Bulb, Leaf	Ethanol extracts	*Curvularia lunata*
Spearmint (*Mentha spicata* Linn.), Greek Sage (*Salvia fruticosa* Mill.), *Thymbra* spp.	Leaf	Essential oils	*Rhizoctonia solani*, *Sclerotium sclerotiorum*
Spanish flag (*Lantana camara* Linn.)	Leaf	Crude Extracts	Castor grey rot (*Botrytis ricini*)
Neem/Margosa (*Azadirachta indica* A.Juss.),	Seed, Leaf	Crude Extracts	Early blight of tomato
Madar (*Calotropis procera* (Ait.) R.Br.	Leaf	Crude Extracts	Tikka leaf spot disease of groundnut

Gurjar *et al.*,2012

Botanicals produced by plants having antimicrobial activity

Madar (*Calotropis procera* (Ait.) R.Br.	Leaf	Crude Extracts	Tikka leaf spot disease of groundnut
Neem/Margosa (*Azadirachta indica* A.Juss.),	Seed	NSKE	Powdery mildew of pea
Spanish flag (*L.camara* Linn.), Pongam (*Pongamia pinnata* L.Pierre.)	Leaf	Crude extracts	Leaf blight of onion
Holy basil (*Oscimum sanctum*Linn.), peach (*Prunus persica* Linn.) Stokes.	Leaf	Essential oil	Grey mould (*Botrytis cinerea*) of grapes
Neem/Margosa (*Azadirachta indica* A.Juss.),	Leaf	Achook formulations (azadirachtina)	Sheath blight of rice
Neem/Margosa (*Azadirachta indica* A.Juss.),	Seed kernel	Neem oil	Rice tungro virus
Neem/Margosa (*Azadirachta indica* A.Juss.),	Leaf, Seed	Achook, Neemazal,	Bacterial blight of rice
Oregano (*Origanum hercleoticum* (weed species)	Leaf	Essential oils	*Fusarium oxysporum*, *Phoma tracheiphila*
Neem/Margosa (*Azadirachta indica* A.Juss.), Black cumin (*Nigelia sativa* Linn. Asfetida (*Ferula asafoetida* Linn.)	Seeds	Essential oils	*Fusarium oxysporum*, *A.niger*, *A.flavus*
Strawberry (*Fragaria* spp.)	Fruit	Volatile compounds	Anthracnose of strawberry
Raspberry (*Rubus* spp.) and Strawberry (*Fragaria* spp.)	Fruit	Volatile compounds	Post harvest decay fungi

Gurjar *et al.*,2012

Botanicals produced by plants having antimicrobial activity

Garden croton (*Codiaeum variegatum* Linn.)	Leaf	Phenolic compounds	*Alternaria alternate*, *Fusarium oxysporum*
Oleander (Nerium *oleander* Linn.)	Leaf	Crude extracts	Brown spot of rice (*Bipolaris oryzae*)
Indian aloe (*Aloe barbadensis* Mill.) Neem/Margosa (*Azadirachta indica* A.Juss.), Tobacco (*Nicotiana tabacum* Linn.)	Leaf	Crude extracts	Dry rot of yam *F. oxysporum*, *A.nizer*
Black pepper (*Piper nigrum* Linn., Clove (*Syzygium aromaticum* (Linn.) Merr. & Perry, Geranium (*Pelargonium graveolens* L'Herit), Nutmeg (*Myristica fragrans* Houtt.), (*Origanum vulgare* spp. *hirtum* (Link) Letsw. and thyme [*Thymus vulgaris* Linn.	Leaf	Volatile oil	Anti bacterial (gram positive and gram negative)

Gurjar *et al.*,2012

Plants containing antimicrobial activity[a]

Common name	Scientific name	Compound	Class	Activity[d]	Relative toxicity[b]
Thyme	*Thymus vulgaris*	Caffeic acid Thymol Tannins —	Terpenoid Phenolic alcohol Polyphenols Flavones	Viruses, bacteria, fungi	2.5
Tree bard	*Podocarpus nagi*	Totarol Nagilactone	Flavonol Lactone	*P. acnes*, other gram-positive bacteria Fungi	
Tua-Tua	*Jatropha gossyphiifolia*	?		General	0.0
Turmeric	*Curcuma longa*	Curcumin Turmeric oil	Terpenoids	Bacteria, protozoa	
Valerian	*Valeriana officinalis*	Essential oil	Terpenoid	General	2.7
Willow	*Salix alba*	Salicin Tannins Essential oil	Phenolic glucoside Polyphenols Terpenoid		
Wintergreen	*Gaultheria procumbens*	Tannins	Polyphenols	General	1.0
Woodruff	*Galium odoratum*	—	Coumarin	General Viruses	3.0
Yarrow	*Achillea millefolium*	?		Viruses, helminths	2.3
Yellow dock	*Rumex crispus*	?		*E. coli*, *Salmonella*, *Staphylococcus*	1.0

[b] 0, very safe; 3, very toxic.
[c] Table is based on data compiled from references 58 and 224
[d] "General" denotes activity against multiple types of microorganisms (e.g., bacteria, fungi, and protozoa), and "bacteria" denotes activity against gram-positive and gram-negative bacteria.

Cowan,1999

The characteristics of effective anti-microbial compounds

- Anti-microbial peptides exhibit all of these properties:

1. Selective toxicity: The ability to sense the difference between self and non-self.
2. Fast killing: Acts in less time than it takes for the invader to multiply.
3. Broad spectra: Should be effective against multiple strains of bacteria, virus and fungus.
4. No resistance development.

Expression of antimicrobial proteins

- Antimicrobial peptides (AMPs) with α-helical structures are ubiquitous and found in many organisms.
- AMPs have been isolated from frogs, insects, and mammalian phagocytic vacuoles (Biggins and Sansom,1999; Tossi *et al.*,2000).
- AMPs are selective for prokaryotic membranes over eukaryotic membrane due to the predominantly negatively charged phospholipids in the outer leaflet of the prokaryotic membrane.
- Such preference is considered a regulatory function in target selectivity.

AMPs with specific actions on bacteria

- While most anti-microbial peptides exhibit a broad range of activity, some AMPs also exhibit some target specificity and can also be classified according to target organism or molecule. e.g.

AMPs /Target Bacteria

- Tachyplesin (Gram -/+)
- Mytilin (Gram +)
- Polyphemusins (Gram -/+)
- Protegrins (Gram -/+)

Genes encoding antimicrobial peptides

- Genes encoding antimicrobial peptides are widely conserved among multicellular organisms, including invertebrates and vertebrates.
- Over 500 antimicrobial peptides have been identified.

Distribution of naturally occurring antimicrobial peptides

Bioactivators

- **Insect peptides:** Insects also posses AMPs called defensins.
- **Amphibian peptides**: Frog skin has been used for medicinal purposes because of the presence of antimicrobial and hemolytic peptides in the skin.
- **Plant peptides:** Thionins are toxic towards both gram-positive and gram-negative bacteria, fungi, yeast, and various mammalian cell types.
- **Other antimicrobial peptides** were isolated which were found to be structurally related to insect and mammal defensins and have been named "plant defensins".
- **Plant defensins have a high antifungal activity.**
- **Bacterial peptides:** Bacteriocins, bacteriocin-like inhibitory substances (BLIS).

Distribution of naturally occurring antimicrobial peptides

Bioactivators

- **Bacterial and fungal peptides:**
- Potential bioactive compounds of interest in the pharmaceutical industry.
- They are usually produced by aerobic spore-forming bacteria in the genera *Bacillus* and *Streptomyces* and in the Fungi *Penicillium* and *Cephalosporium*.
- However, bioactive compounds with effectiveness against antibiotic resistant strains of bacteria are more interested.
- Bioactive compounds such as Micacocidin A, B, C antimycoplasmal) and C-14, a cyclic dipeptide (chitinase inhibitor) have also been reported from marine *Pseudomonas* species.

Okulate,2009;Suresh,2013;..

Distribution of naturally occurring antimicrobial peptides

Bioactivators

- **Bacterial peptides:**
- Probably 99% of all bacteria generate at least one bacteriocin, while both gram-positive and gram-negative bacteria are capable of producing the bacteriocins.
- Many phytopathogenic bacteria including members of the Corynebacterium, *Erwinia*, *Pseudomonas*, *Xanthomonas* and *Agrobacterium* produce bioactive bacteriocins.
- These bacteriocins are highly specific, cost effective and are even eco-friendly.

Jabeen,2007

Examples of primary amino acid sequences of natural antimicrobial peptides

Peptide	Structure
Gramicidin S	Cyclic (LOVPF^{d}LOVPF^{d})
Bacitracin	Cyclized I (C)LE^{d}I (KO^{d}IFHD)D^{d}-NH_2
Polymyxin B (a peptide antibiotic)	Cyclized isooctanoyl BTBB (BF^{d}LBBT)
defensin (NP-1)	$VVC_1AC_2RRALC_3LPRERRAGFC_3RIRGRIHLC_2$ C_1RR
defensin 1	$DHYNC_1VSSGQC_2LYSAC_3PIFTKIQGTC_2YRGKAKC_1C_3K$
Crab tachyplesin	$RRWC_1FRVC_2YRGFC_2YRKC_1R$
Cattle bactenecin	$RLC_1RIVVIRVC_1R$
Silk moth cecropin A	KWKFKKIEKMGRNIRDGIVKAGPAIEVIGSAKAI
Cattle indolicidin	ILPWKWPWWPWRR
Bacterial nisin	$IXA_1IULA_1Z_2PGA_2KZ_3GLAMGA_3NMKZ_4AZ_5A_4HA_5SIHVUK$

Hancock and Chapple,1999

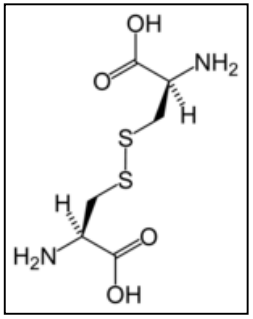
Classes of antibacterial peptides and proteins

Cationic peptides

- Antimicrobial cationic peptides are thought to be an important component in host defense responses against pathogenic agents.
- There are four structural classes of cationic(positive charge) antimicrobial peptides:

1. The disulfide-linked β-sheet peptides, including the defensins.
2. The amphipathic α-helical peptides such as the cecropins and melittins.
3. The extended peptides, which often have a single amino acid predominating (e.g. indolicidin).
4. The loop-structured peptides like bactenecin.

Cationic peptides
Structures



Disulfide bonds in proteins

- On the basis of their structural features, cationic peptides can be divided as well into three different classes:

1) Linear peptides forming-helical structures,
2) Cysteine-rich open ended peptides, containing single or several disulfide bridges.
3) Molecules rich in specific amino acids such as proline, glycine, histidine and tryptophan.

Important subfamilies of cationic peptides include:

- Thionins
- Defensins
- Cecropins
- Amino acid-enriched class
- Histone derived compounds
- Beta-hairpin
- Magainins

Antimicrobial peptides from plants

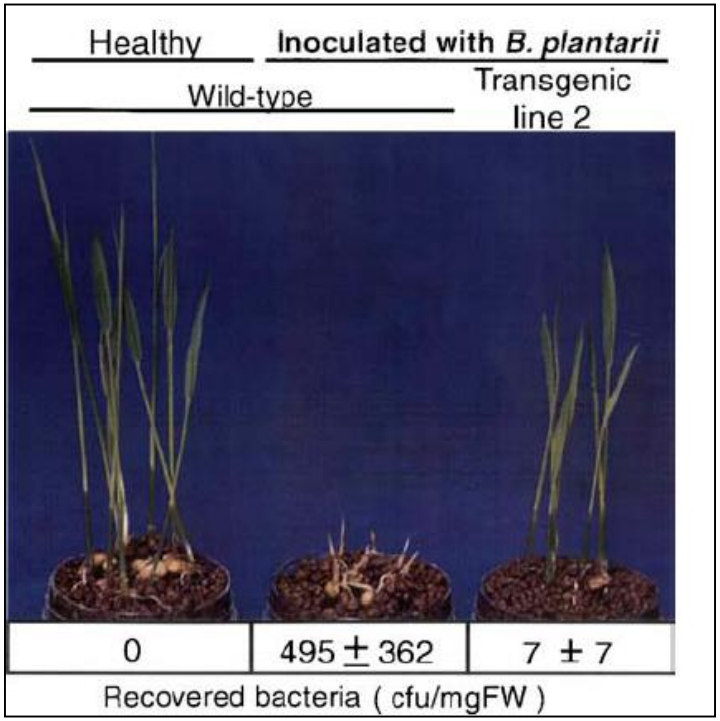
- Various types of antimicrobial peptides have been identified in plants, including:
- Thionins,
- Maize zeamatin,
- Coffee circulin, and
- Wheat puroindoline.
- Antimicrobial peptides from plants harbor all other antimicrobial peptides structural features plus 3 or 4 disulfide bonds, such as thionin from barley (*Hordeum vulgare*) and plant defensin from radish (*Raphanus sativus*).

Antibacterial agents from plants
Thionins

- Thionins are plant antimicrobial proteins which are able to inhibit a broad range of pathogenic bacteria *in vitro*.
- Carmona *et al*.,1993 reported the expression of alpha-thionin gene from barley in transgenic tobacco confers enhanced resistance to two pathovars of *P. syringae*.
- Unfortunately, most thionins can be toxic to animal and plant cells and thus may not be ideal for developing transgenic plants.
- Based on the first positive results obtained in transgenic tobacco against *P. syringae* pv. *tabaci*, lipid transfer proteins and snakins may be good candidates for use against some plant pathogenic bacteria.

Transforming rice with a thionin gene against *Burkholderia plantarii*

- The effectiveness of transforming rice with a thionin gene from oats in protecting the plant from infection by *Burkholderia plantarii*:
- Uninoculated controls on the left,
- inoculated wild-type plants in the centre, and
- inoculated transformants on the right.
- Numbers below the pots are colony-forming units of the bacterium/mg fresh weight of plant material recovered from the plants.



Strange,2003

Antibacterial agents from plants
Defensins

- Plant defensins (PDFs) from radish (Rs-AFP1, 2, 3, 4), which share structural features with insect and mammalian defensins (which have 3 disulfide bonds), are small, cysteine-rich peptides consisting of 45-54 amino acids with 4 disulfide bonds.
- They are conserved in several plant species, including members of the Brassicaceae, and inhibit the growth of a broad range of microbes, but do not appear to be toxic to mammalian and plant cells.
- Rice plants do not contain these peptides.

Genetic engineering for disease resistance in Rice using antimicrobial peptides

- A gene family of plant defensins (AFP) is conserved in several plant species, including those of the Brassicaceae.
- Rice plants do not contain these peptides.
- AFP1 homologs in 8 Brassicaceae vegetables have been identified, and their structural differences have been determined.
- AFP1 gene variants from *Brassica oleracea* and *B. campestris* conferred an effective resistance to both rice blast and bacterial leaf blight.
- The results of *in vitro* and *in vivo* analyses suggest that plant defensins have the potential to enhance broad-spectrum disease resistance in rice through genetic engineering.
- The modification of the defensin genes led to an increase in the broad disease resistance spectrum.

Antibacterial activity of defensin genes

- Transgenic rice plants expressing genes for pathogenesis-related (PR) protein, for example thionin, chitinase, and puroindoline, show a high level of resistance to rice blast or bacterial leaf blight.
- Plants that constitutively expressed a plant defensin gene from *B. oleracea* or *B. campestris* were tested for resistance to rice blast and bacterial leaf blight.

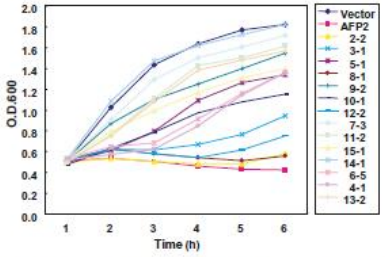


Fig. 2. Antimicrobial activity against *E. coli* of defensin peptides from *B. campestris* with single amino acid substitutions

Fig. 3. Resistance to rice blast disease in transformant by introduction of defensin gene
A: Non-transformant. B: Transformant. C: Non-transformant, not infected.

Fig. 4. Resistance to bacterial leaf blight in transformants by introduction of defensin gene
A: Transformant by introduction of modified defensin gene.
B: Transformant by introduction of native defensin gene. C: Non-transformant.

Kawata *et al.*,2003

Genetic engineering for disease resistance in **Banana** using antimicrobial peptides

- The transgenic banana plants resistant for bacterial wilt disease (*Xanthomonas campestris* pv. *musacearum*, Xcm) can be developed by using genes for antimicrobial peptides, and other plant defense-related proteins that tend to act as bactericidal compounds.

Antibacterial agents from insects
Defensins

- A group of cecropin-like insect defensins has been purified from flesh fly (*Sarcophaga peregrina*), which includes three families of sarcotoxins and the family of sapecins (Natori,1994).
- Sarcotoxins are active against a wide range of Gram negative bacteria at submicromolar concentrations but not toxic to tobacco and rice suspension cells at up to 25 mM.
- The gene (sarco) coding for sarcotoxin IA has been introduced into potato by *Agrobacterium* mediated transformation and yeast.
- The recombinant sarcotoxin from yeast showed toxic effects to *E. carotovora*, *P. syringae* pv. *lachrymans* and *R. solanacearum*, which was similar to the effects of the native sarcotoxin.

Antibacterial agents from insects
Cecropins

- The best-known antibacterial peptides of insect origin are cecropins, which accumulate in the haemolymph of the:
- Giant silkmoth (*Hyalophora cecropia*),
- Silkworm (*Bombyx mori*), and
- Drosophila as a response to infection.
- These short, linear peptides (31-39 amino acids, aa) interact with the outer phospholipid membranes of both Gram-negative and Gram-positive bacteria and modify them by forming a large number of transient ion channels.

Antibacterial agents from insects
Cecropins

- Native (cecropin B), mutant (SB37=38 aa, MB39=39 aa) and synthetic (Shiva-1 peptide=38 aa, D4E1=17 aa) cecropins are active *in vitro* against a wide range of plant pathogenic bacteria including *Erwinia amylovora*, *P. carotovorum* subsp. *carotovorum* and *P. atroseptica*, *Erwinia chrysanthemi*, *Pseudomonas syringae* several pathovars, *R. solanacearum* and *X. campestris* several pathovars.
- They exert no toxicity at bactericidal concentrations to cultured cells or protoplasts of several plant species.
- Therefore, cecropins have been considered as potential candidates to protect plants against bacterial pathogens.

Antibacterial agents from insects
Attacins

- The same insects that synthesize sarcotoxins also produce attacins, which belong to another family of six 20 kDa antibacterial proteins, in response to bacterial infection.
- Attacins alter the structure and permeability of prokaryotic membranes by binding to lipopolysaccharide in the bacterial envelope and inhibiting the synthesis of the outer membrane proteins.
- Increased *in vitro* and greenhouse resistance to fire blight caused by *E. amylovora* by the expression of the attacin E gene (attE) in transgenic apple plants was reported by Norelli *et al.*,1994.
- The data obtained with these two constructs indicate that attacin can enhance resistance to *E. amylovora*.

Antibacterial agents from insects
Attacins

- As with cecropin, have shown attacin activity in transgenic potato to *E. carotovorum* ssp. *atrosepticum*.
- Chen and Kuehnle (1996) have also demonstrated the expression of attacin in calli induced from transformed *Anthurium*.
- A number of distinct, antibacterial peptides or proteins have been described in other insects.

Antibacterial agents from higher animals and mammals

Esculentin

- The best-known peptides are the pore-forming magainins, bombinins, brevinins, esculentin, rugosins and temporins isolated from frog skin.
- Of these, only magainin analogs (MSI-99 and Myp30) and esculentin have recently been transferred successfully into plants for use against bacteria including *P. carotovorum* ssp. *atrosepticum*, *P. syringae* pv. *tabaci* in transplastomic tobacco and to *P. carotovorum* ssp. *atrosepticum* in transgenic tobacco.
- Indolicidin has been expressed in transgenic tobacco and is currently tested in the field for resistance to *P. syringae* pv. *tabaci*.

Antibacterial agents from frog
Magainins

- Magainin is a defense peptide secreted from the skin of the African clawed frog (*Xenopus laevis*), first discovered by Zasloff (1987).
- Magainins and their analogs have been studied as:
- A broad-spectrum topical agent,
- A systemic antibiotic,
- A wound-healing stimulant, and
- An anticancer agent (Jacob and Zasloff,1994).
- However, only magainin analogs (MSI-99 and Myp30) have recently been transferred into plants for used against bacteria.

Antibacterial agents from frog
Magainins

- Li *et al*., 2001 have reported disease resistance, to both a fungal and a bacterial pathogen, conferred by expression of a magainin analog, Myp30, in transgenic tobacco (*Nicotiana tabacum* var. Petit Havana).
- Another analog MSI-99, when expressed in tobacco via chloroplast transformation conferred both *in vitro* and *in planta* resistance to plant pathogenic bacteria and fungi.

Magainin 2

An antimicrobial peptide against *P.s.* pv. *tabaci*

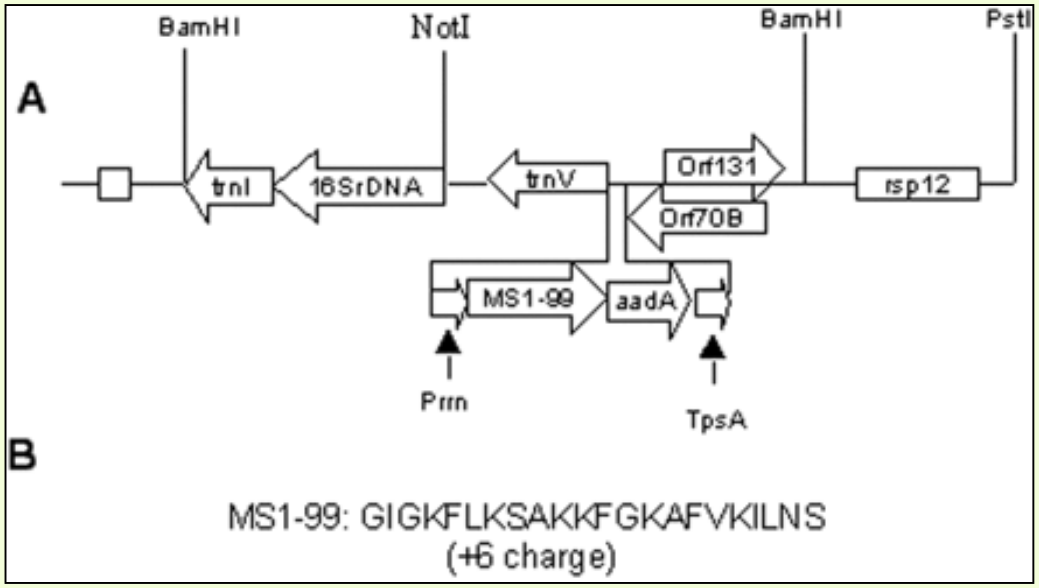
- The AMP used in this study (MSI-99) is an analog of magainin 2, a defense peptide secreted from the skin of the African clawed frog.
- Chloroplast transformation was selected because of several advantages over nuclear transformation.
- When it was expressed via the chloroplast genome to obtain high levels of expression in transgenic tobacco plants.

DeGray *et al.*,2001

Transformation vector and MSI-99 peptide sequence

A. Vector contains a selectable marker gene (*aadA*) that confers resistance to spectinomycin, 16S *rrn* promoter, *psbA* terminator, and petunia chloroplast DNA flanking sequences.

B. Amino acid sequence of the AMP MSI-99.



DeGray *et al.*,2001

In vitro assay

Minimum inhibitory concentrations(μg ml^{-1}) of magainin 2 and MSI-99

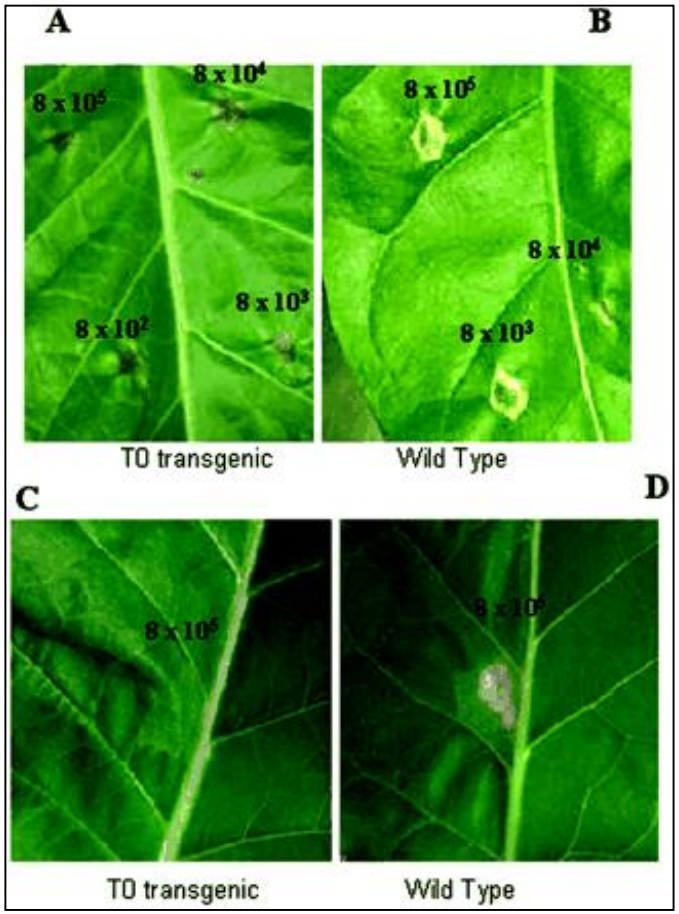
Phytopathogens	MSI-99	Magainin 2
P. syringae	1	32
Erwinia carotovora	1	32
Phytophthora parasitica	64	>256
Fusarium solani	2	4
Fusarium graminearum	4	4
Thielaviopsis basicola	4	8
Botrytis cinerea	8	16

Minimum Inhibitory Concentrations(MICs) required to inhibit growth after 24 h. Based on 1,000 fungal conidiospores or 1,000 bacterial cells. MSI-99 was most effective against *Pst*, requiring only μg mL^{-1} of MSI-99.

DeGray *et al.*,2001

In planta bioassays

- Five- to 7-mm areas of TO transformants and non-transformed tobacco cv Petit Havana leaves were scraped with fine-grain sandpaper.
- Ten microliters of 8 ×10^5, 8 × 10^4, 8 × 10^3, and 8 × 10^2 cells from an overnight culture of *P. syringae* pv *tabaci* were added to each prepared area.
- Photos were taken 5 d after inoculation.
- A, Transgenic leaf; B, wild-type leaf; C, transgenic; D, wild type were injected with 25 µl of 8 × 10^3 cells of *Pst*.
- Pictures were taken 5 d after inoculation.
- Transformed leaf show only slight discoloration, whereas the wild-type leaf shows necrosis.



DeGray *et al.*,2001

Antibacterial proteins

Lysozymes

- Lysozymes are a ubiquitous family of enzymes that occur in many tissues and secretions of humans, animals, as well as in plants, bacteria and phage.
- Lysozyme is a monomeric peptide, it is not classified as an antimicrobial peptides because it is relatively large (148 aa).
- The lysozyme attacks the murein layer of bacterial peptidoglycan resulting in cell wall weakening and eventually leading to lysis of both Gram negative and Gram-positive bacteria.
- Hen egg-white lysozyme (HEWL), T4 lysozyme (T4L), T7 lysozyme, human and bovine lysozyme genes have been cloned and transferred to enhance plant bacterial or fungal resistance.

Antibacterial proteins

Lysozymes

- Those from egg white and bacteriophage have been studied and there are now reports of proteins with similar activity from plants but, in addition, these enzymes have chitinase activity.
- The lysozyme genes have been used to confer resistance against plant pathogenic bacteria in transgenic tobacco plants, found to inhibit the growth of *P. syringae* pv. *tabaci*.
- T4L, from the T4-bacteriophage, also has been reported to enhance resistance of transgenic potato against *E. carotovora*, which causes bacterial soft rot.
- Transgenic apple plants with the T4L gene showed significant resistance to fire blight infection (Ko, 1999).

Antibacterial proteins

Antibodies

- An interesting strategy for control of bacteria may be the expression in transgenic plants of antibodies that target specific bacterial pathogenicity factors.
- Specific binding of antibodies to one or more bacterial factors, such as secreted lytic enzymes or extracellular proteins, is expected to compromise bacterial pathogenicity.

Antibacterial proteins

Antibodies

- However, the recently developed phage display technique offers a more convenient and faster means to produce single-chain variable antibody fragments (scFv's).
- This method is based on:
- Bacterial recombination and gene expression techniques and also allows the ready isolation of the gene encoding for the desired antibody.

Antibacterial proteins
Antibodies

- For the antibody strategy to be successful, antibodies must be correctly expressed and secreted in plants.
- It should be stressed, however, that transgenes encoding antibodies, just like many other foreign genes, can be inactivated by various mechanisms in the plant, such as gene silencing.
- To my knowledge, this strategy has not been applied for transgenic control of plant pathogenic bacteria though scFv's against *R. solanacearum* has been produced by phage display.

Amino acid-enriched class

Polygalacturonase-inhibiting proteins (PGIPs)

- A number of polygalacturonase-inhibiting proteins (PGIPs) have been isolated from dicotyledonous plants and they, in common with resistance genes, possess leucine rich repeat domains.
- They are thought to contribute to resistance to fungal pathogens.
- These are highly active elicitors of defence responses such as the hypersensitive response, lignification and the synthesis of phytoalexins.
- Not surprisingly, increased plant resistance is being sought in transgenic plants over-expressing PGIPs

Transgenic crops

- Plants are genetically engineered to:
- Resist the effects of weed killers (herbicides).
- Tolerate salt in the soil.
- Resist insect pests (produce *Bt* toxins from *Bacillus thuringensis*).
- Produce pharmaceuticals and vaccines.
- Therefore, most of the transgenic crop varieties currently grown by farmers are either herbicide tolerant or insect pest-resistant.
- In addition to the crops listed below, minor acreages were planted to transgenic potato, squash, and papaya.

Worldwide production area of transgenic crops and traits

Crop	1999 (millions of acres) inplanted areas
Soybean	53.4
Corn	27.4
Cotton	9.1
Canola	8.4
Potato	0.3
Squash	0.3
Papaya	0.3
Trait	
Herbicide tolerance	69.4
Bt insect resistance	22
Bt + herbicide tolerance	7.2
Virus resistance	0.3

Transgenic crop production area

Country	2000 (millions of acres) inplanted areas	Crops grown
USA	74.8	soybean, corn, cotton, canola
Argentina	24.7	soybean, corn, cotton
Canada	7.4	soybean, corn, canola
China	1.2	cotton
South Africa	0.5	corn, cotton
Australia	0.4	cotton
Mexico	minor	cotton
Bulgaria	minor	corn
Romania	minor	soybean, potato
Spain	minor	corn
Germany	minor	corn
France	minor	corn
Uruguay	minor	soybean

Genetic Engineering Plants

8. plantibody-mediated plant disease resistance

Plant biotechnology

Plant transformation/transgenic plant production

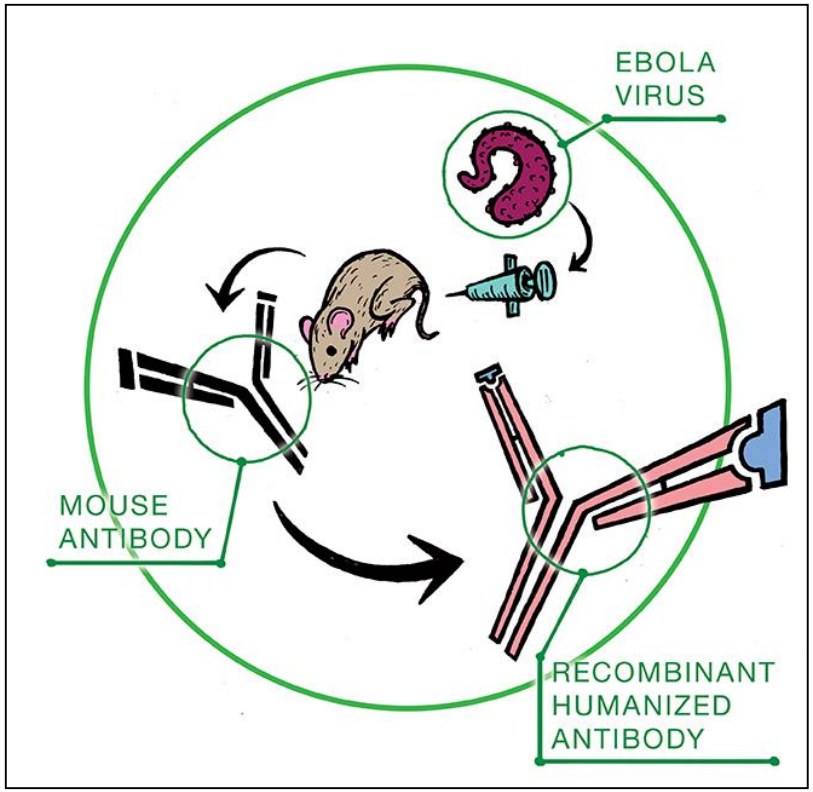
Enhancing resistance with plantibodies

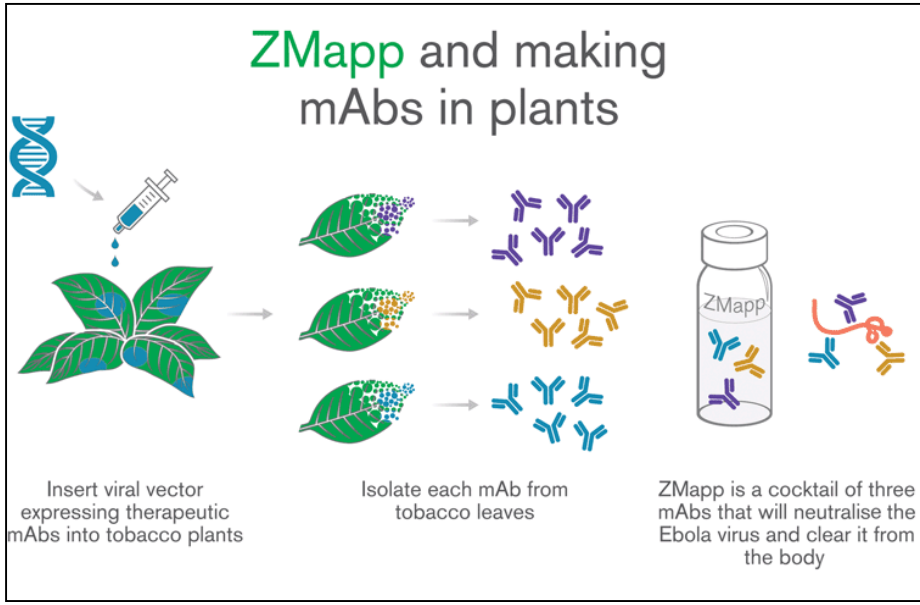
- The term "plantibody" refers to an antibody that has been produced by genetically engineered plants.
- Plantibodies are the important part of adaptive immune system.
- Plants do not naturally make the antibodies; but, they can be produced in plants by introducing antibody coding genes from humans and animals.
- Plant derived antibodies are called as plantibodies and known to work in the same way as mammalian antibodies.

Ethan Novena, Prasad *et al.*,2017

Plant biotechnology

The success story of ZMapp during last year's Ebola outbreak highlights the potential of plant-made monoclonal antibodies (mAbs) as life-saving treatments



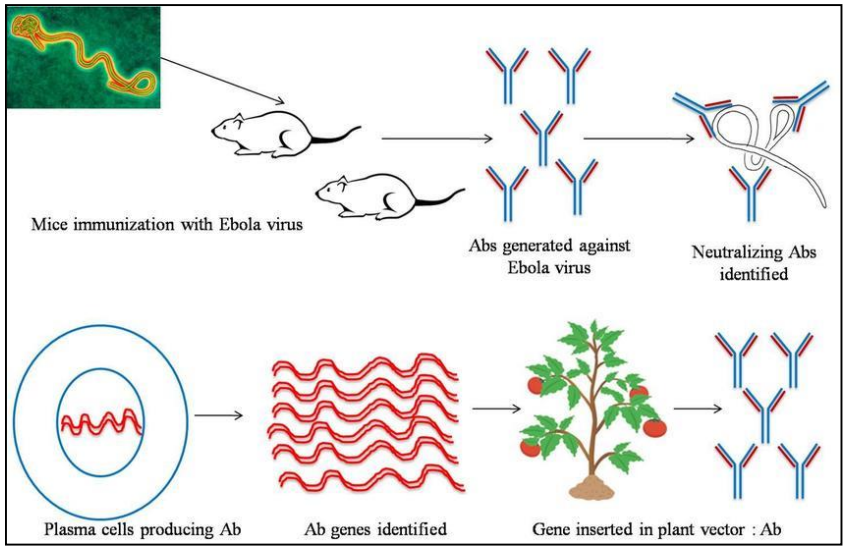


Plant biotechnology

Plant transformation/transgenic plant production

Enhancing resistance with plantibodies

- **Plantibody** = a plant derived antibody.
- Antibodies are an essential part of the immune system of many organisms.
- They recognize viral antigens and other dangerous compounds and signal a response.



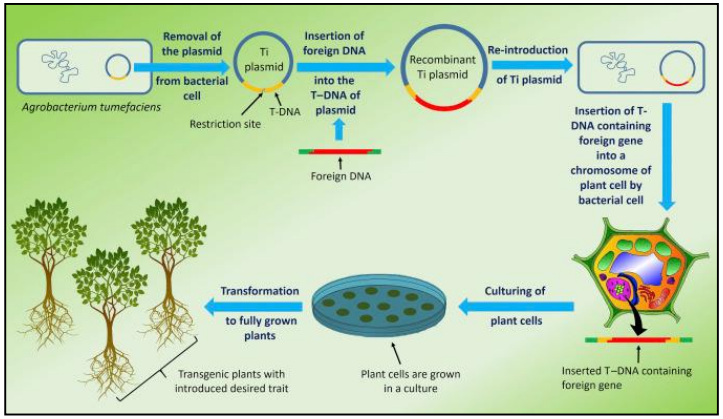
Ethan Novena, Prasad *et al.*,2017

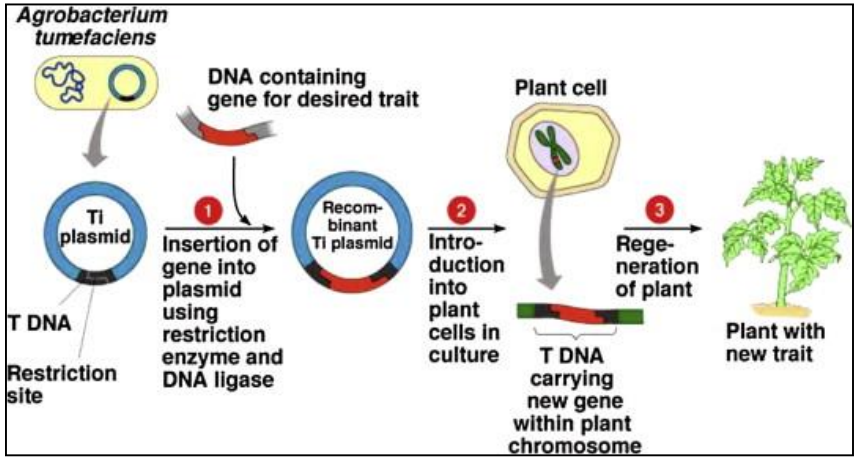
Plant biotechnology

Enhancing resistance with plantibodies

The Transformation Process

- Isolate: DNA from a mammal containing an antibody-coding gene.
- Insert: the gene of interest into Ti-plasmid from *Agrobacterium* by cutting with restriction enzymes and joining with DNA ligase.
- Introduce: the recombinant Ti plasmid to plant cells in culture. DNA segment of interest is transferred to the plant chromosome.
- Regenerate: the transgenic plant in vitro or farms, greenhouses, etc.





Ethan Novena;..

Plant biotechnology

Enhancing resistance with plantibodies

Plantibodies targeting phytoplasmas

- Phytoplasmas are normally controlled by the breeding and planting of disease-resistant crop varieties (perhaps the most economically viable option) and by the control of insect vectors.

1. Tissue culture can be used to produce healthy clones of phytoplasma-infected plants.
2. Cryotherapy (i.e., the freezing of plant samples in liquid nitrogen) prior to tissue culture increases the probability of producing healthy plants in this manner.
3. Plantibodies targeting phytoplasmas have also been developed.

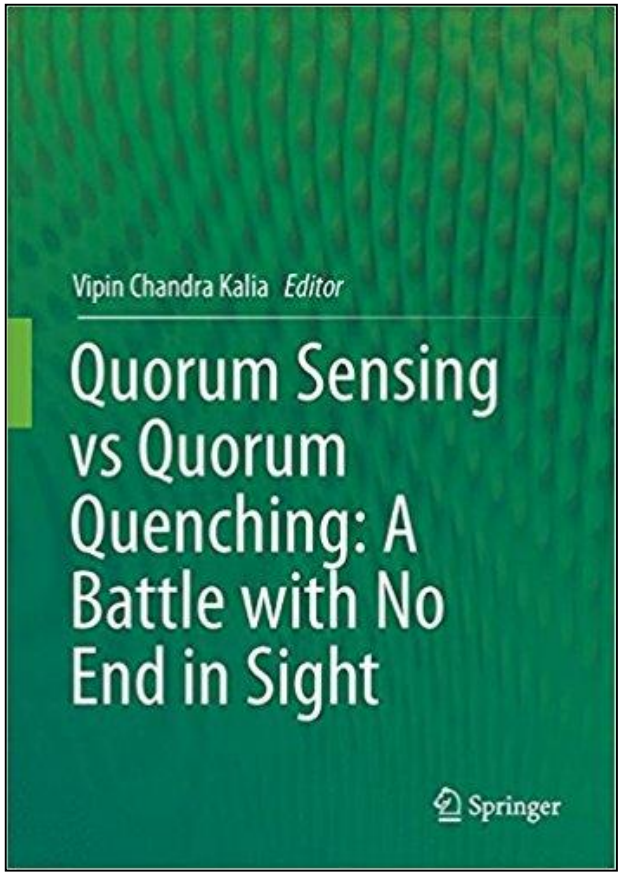
Wikipedia,2019

Anti-quorum sensing
Quorum quenching (QQ)

Anti/Qs a novel strategy for controlling pathogenesis of bacteria(confusing bacterial pathogens).

Quorum Sensing vs Quorum Quenching: A Battle with No End in Sight

- **Quorum Sensing vs Quorum Quenching: A Battle with No End in Sight.**
- by Vipin Chandra Kalia (Editor).
- Springer; 2015 edition (September 30, 2014)
- 391 pages.



Quorum quenching

A promising approach to prevent bacterium to become pathogenic

Magic bullets

- The anti-QS antagonists may serve as the next generation "magic bullets", but care must be taken that these molecules that are not bactericidal so they may have limited application for immunocompromised patients.
- Various antibiotics or drugs (including salicylic acid used at concentrations ranging from 1 to 30 mM), which decrease the expression of QS-related genes and corresponding virulence factors.
- Perhaps, a cocktail therapy (A mixture of drugs) involving both antibiotics and anti-QS antagonists may provide synergistic effects.

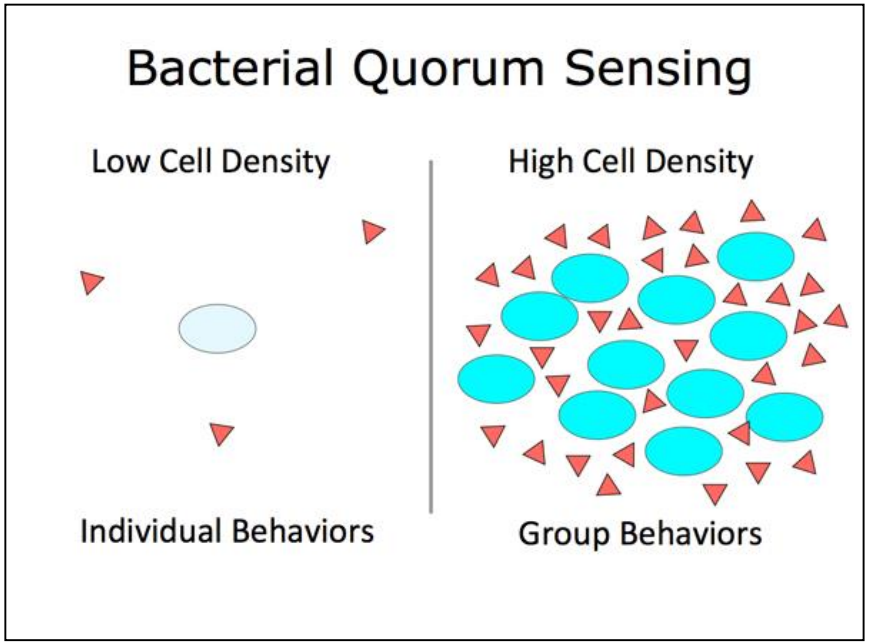
Koh *et al.*,2013; Vandeputte,2014

Quorum sensing

The process of cell-cell communication in bacteria

The social lives & behaviors of microbes

- Quorum-sensing, also called autoinduction, describes the phenomenon whereby the accumulation of signaling molecules called autoinducers in the surrounding environment enables a single cell to assess the number of bacteria (cell density) so that the population as a whole can make a coordinated response.



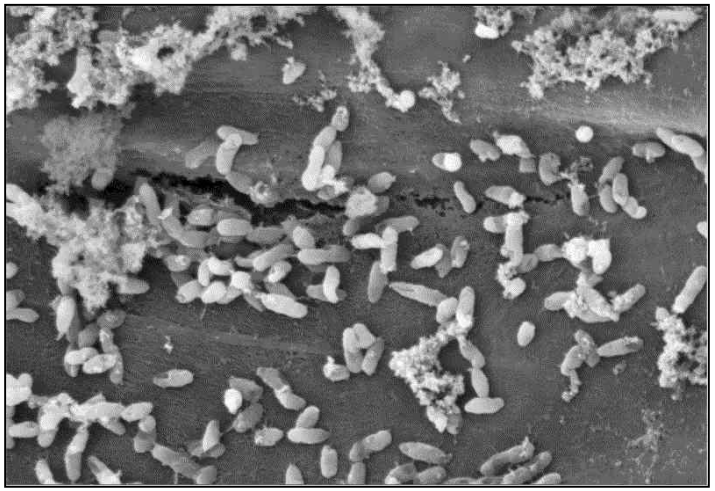
West *et al.*,2007; Bassler Lab Research

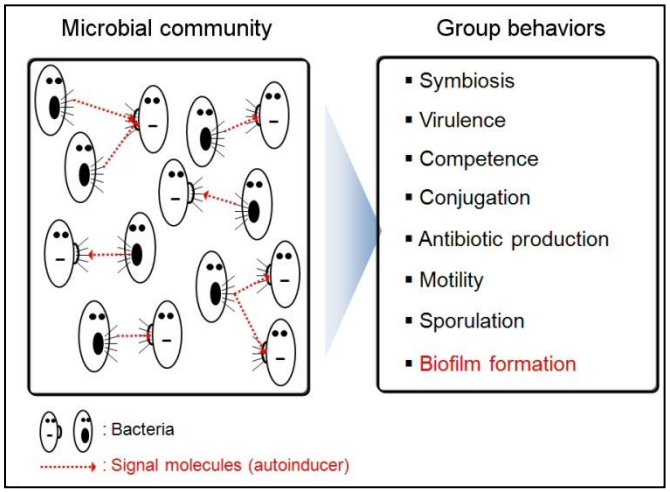
Quorum sensing

Communication between bacteria

The language of Bacteria

- Bacteria live in colonies.
- Most, if not all, bacteria communicate with their neighbors (Small Talk).
- The term 'Quorum Sensing' (QS) to describe the phenomenon whereby the accumulation of signalling molecules enable a single cell to sense the number of bacteria (cell density).



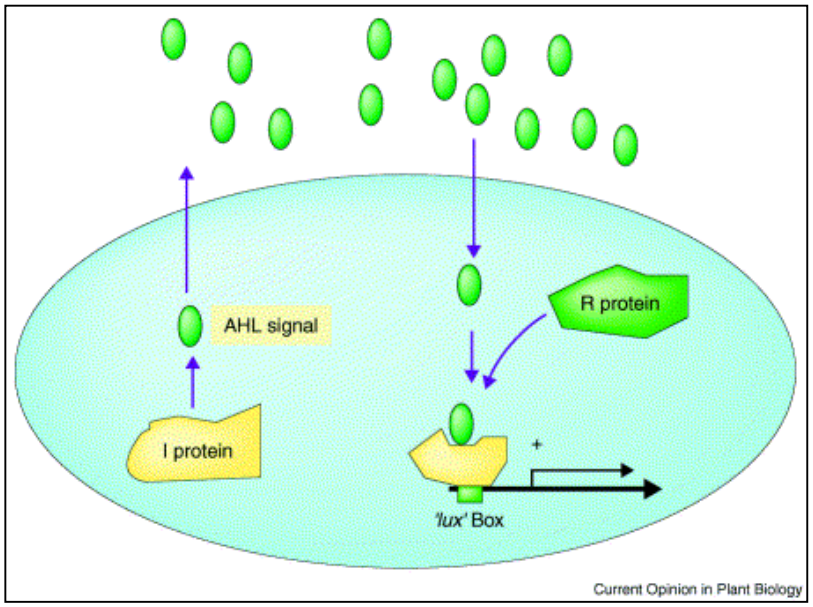


Quorum sensing

Bioluminescence

Transcription of the lux operon

- The AHLs are sensed by proteins belonging to the LuxR family of response regulators.
- LuxR homologues contain two domains:
  1. an AHL binding domain,
  2. a DNA binding domain.
- When AHL is bound, it alters the configuration of the LuxR homologue protein, enabling it to interact with DNA and act as a transcriptional activator.



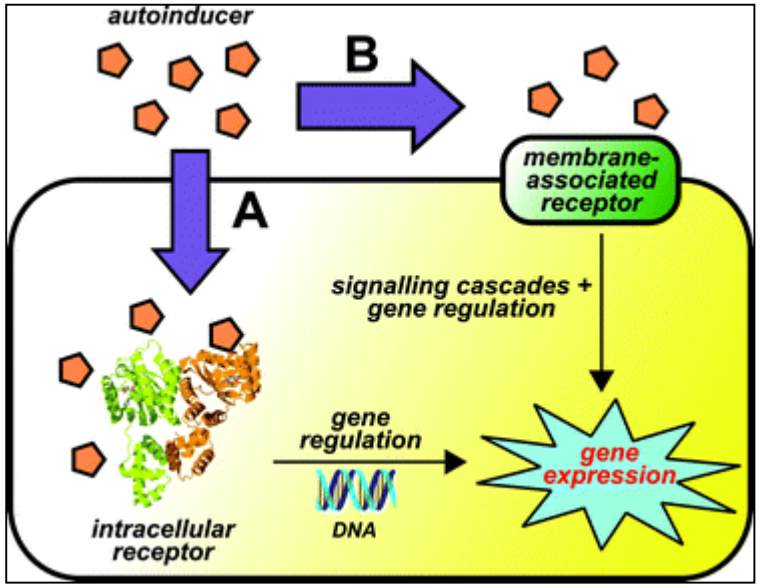
The two key components of the QS system: the *luxI* and *luxR* homologues:

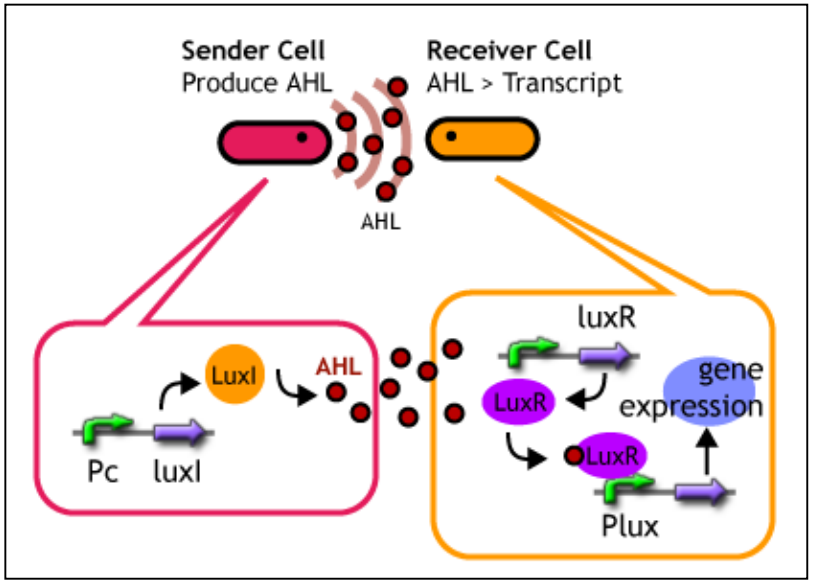
1. *luxI* (signal generator);
2. *luxR* (response generator).

Quorum sensing

Bioluminescence

Transcription of the lux operon



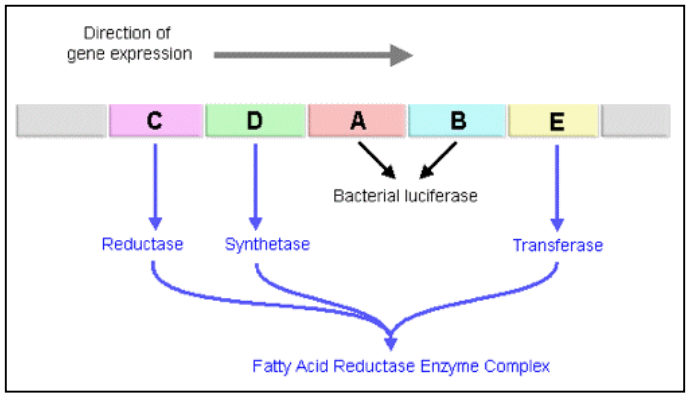


Quorum sensing

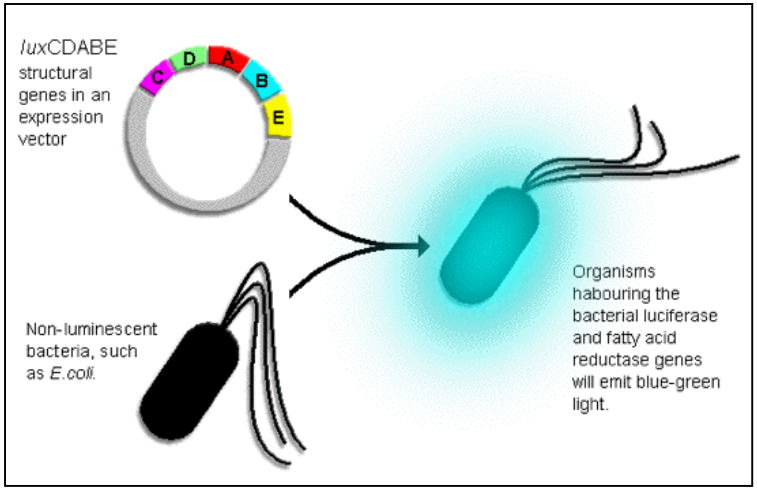
LuxR-type receptor

lux genes expression in mutant *E. coli*

- The insertion of the foreign *lux*CDABE structural genes into the organism allows the fatty acid reductase enzyme complex and luciferase to be expressed, and the function of luciferase confers the organism the ability to emit light.



The arrangement of the *lux*CDABE open reading frames.



Lin and Meighen,2009

Quorum sensing

Bioluminescence

Transcription of the lux operon

- The LuxR AHL complex forms higher clusters, so-called oligomers or polymers.
- These polymers can bind to the DNA (more exactly: to the so-called lux box, which is the promoter region of the lux operon) and act there as transcription factor.

Li and Nair,2012;..

A simplified model for AHL-mediated gene regulation in bacteria

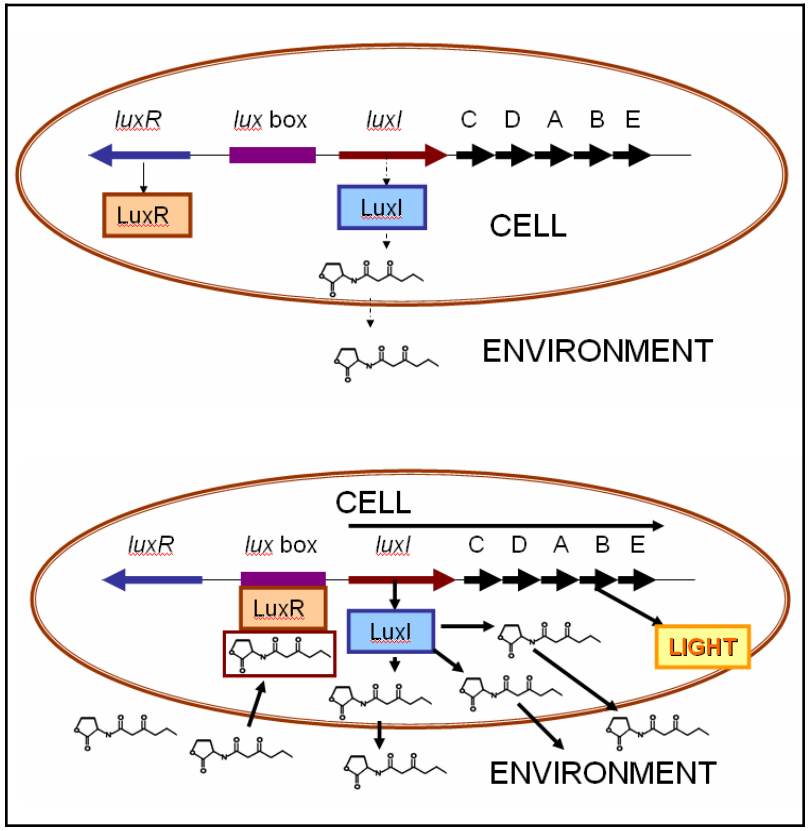
In Gram negative bacteria

Vibrio fischeri

a) When there are few bacteria nearby, the cell produces very little AHL.

b) As the concentration of cells in the surroundings increases, the signal accumulates.

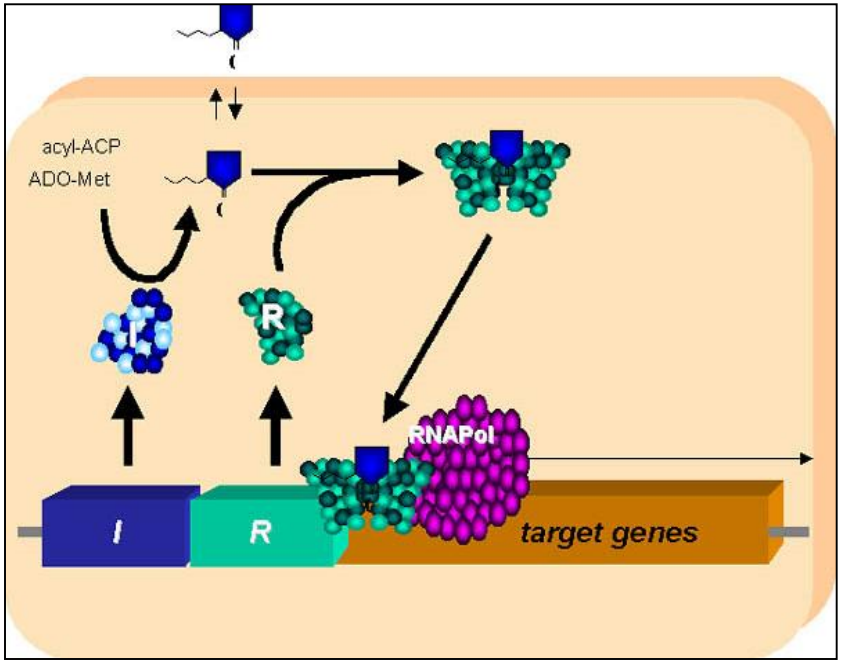
- The AHL binds to LuxR and the LuxR/AHL complex binds to a region of DNA called the lux box.
- This activates the transcription of structural genes whose products give rise to luminescence.
- LuxI production also increases, leading to increased synthesis of the AHL.
- Homologues of the *V. fischeri* LuxR and LuxI proteins have been identified in over 25 species of gram-negative bacteria.



Lal,2009

A simplified model for AHL-mediated gene regulation in bacteria
In Gram negative bacteria

- The genes *I* and *R* represent the genes encoding the AHL synthase and regulatory protein, respectively.
- In the presence of sufficient AHL signal, the R regulatory protein is activated, possibly by dimerization.
- The activated R regulatory protein binds to a specific binding site and stimulates (or represses) transcription initiation by RNA polymerase holoenzyme (RNAP+sigma factor).



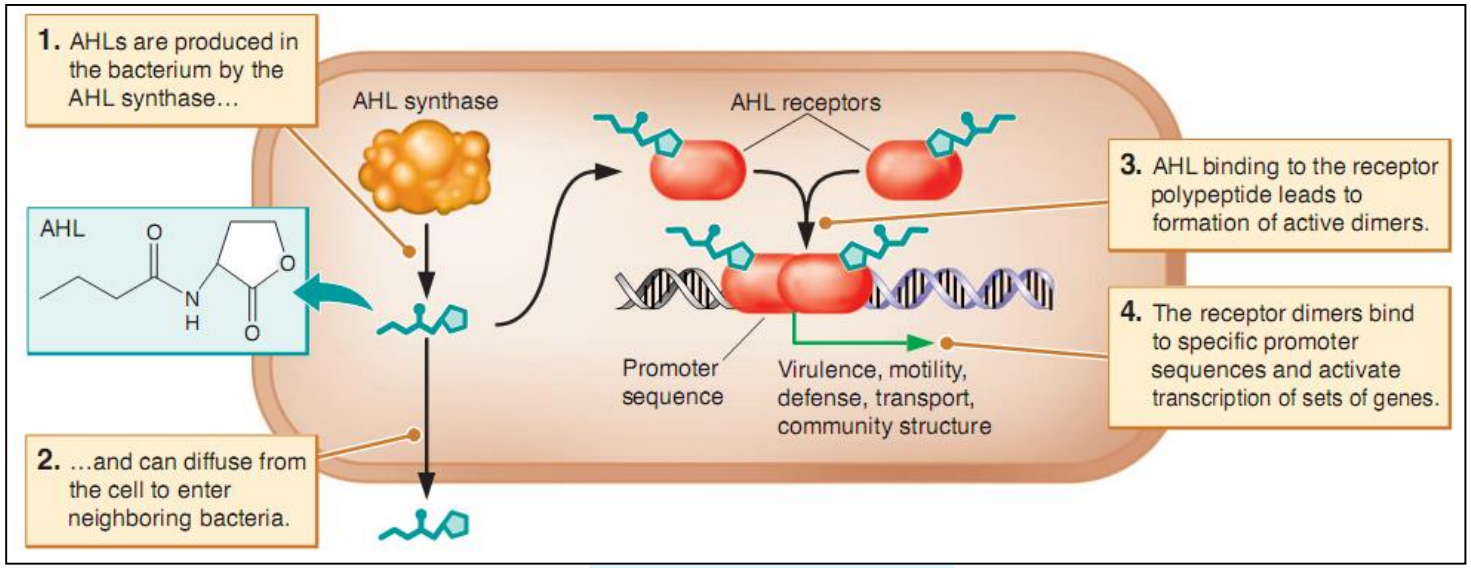
RNA polymerase (RNAP) is the enzymatic machinery responsible for transcription, a key regulatory step in gene expression. The prokaryotic RNAP is a highly conserved, "crab claw" shaped enzyme with a molecular mass of ~400kD.

Pierson III,2005;..

Quorum sensing

Molecular mechanics of AHL-mediated QS

- AHLs are produced in the bacterium can diffuse from the cell to enter neighboring bacteria.
- AHL binding to the receptor polypeptide leads to formation of active dimers.
- The receptor dimers bind to specific promoter sequences in the bacterial genome(lux box) and activate transcription of sets of genes.



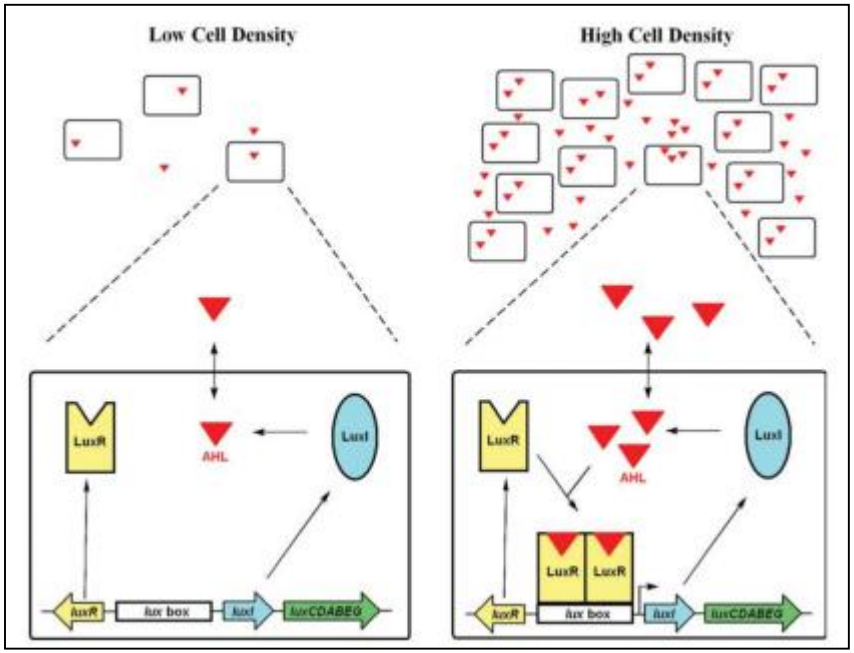
Bauer *et al.*,2005

Quorum sensing

Bioluminescence

The *lux* operon is a 9 kb fragment that consists of 5 genes in *Vibrio fischeri*

- Upon binding to its cognate signal AHL molecule, LuxR in *V. fischeri* binds to a short sequence termed lux box, and activate the transcription of the downstream operon, *luxICDABEG*, which contains the *luxI* gene that encodes the AHL synthase.



Li and Nair,2012

Quorum sensing
Regulate a number of gene expression

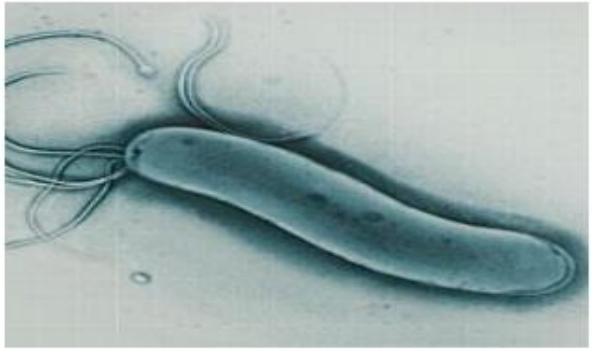
- Quorum sensing enables a bacterial species to sense its own number and regulate gene expression including:

1. Biofilm formation,
2. Expression of virulence factors,
3. Luminescence,
4. Pigment production,
5. Spore formation,
6. Entry into stationary phase
7. Conjugal transfer of plasmid DNA and
8. Mechanisms of resistance to stress conditions, which are of major importance in bacterial pathogenesis.

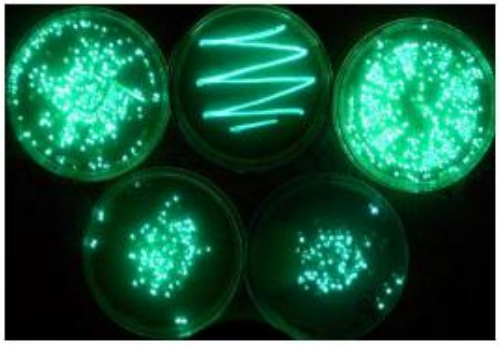
Olivero *et al.*,2011;Schelz,2009

Quorum sensing

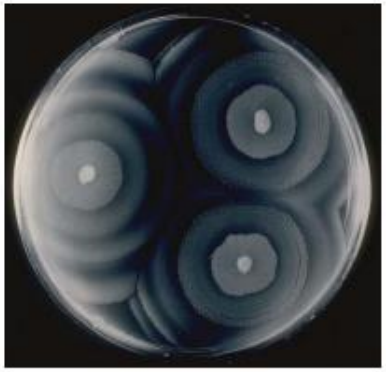
Some other functions



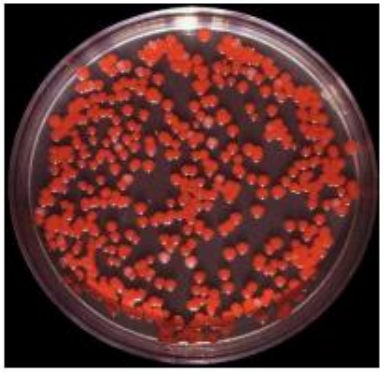
Virulence



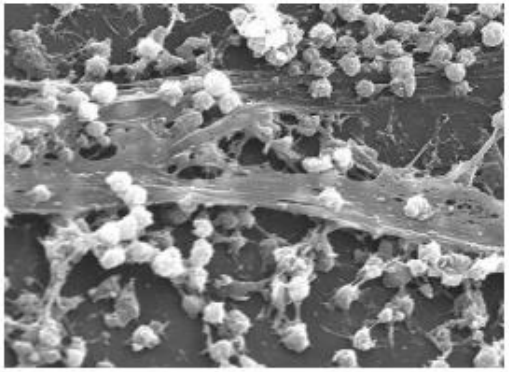
Bioluminiscence



Swarming



Pigments



Biofilms

Quorum sensing

Bacterial hormone-like signal molecules(AHLs)

- Quorum sensing (QS) is mediated by a group of small, diffusible hormone-like signal molecules (e.g. AHLs) produced by the bacteria.
- These molecules are also known as 'quormones' or bacterial pheromones because the bacterial quormones function similarly to insect pheromones.

Martinelli *et al.*,2004;..

Quorum sensing
Chemical communication signals

- QS has been described:

1. Between cells of the same species (intraspecies),
2. Between species (interspecies), and
3. Between bacteria and higher organisms (inter-kingdom).

Diggle *et al.*,2009

Quorum sensing
Interkingdom communications

- Pathogenic bacteria during infection of a host (e.g. humans, other animals or plants) to co-ordinate their virulence in order to:

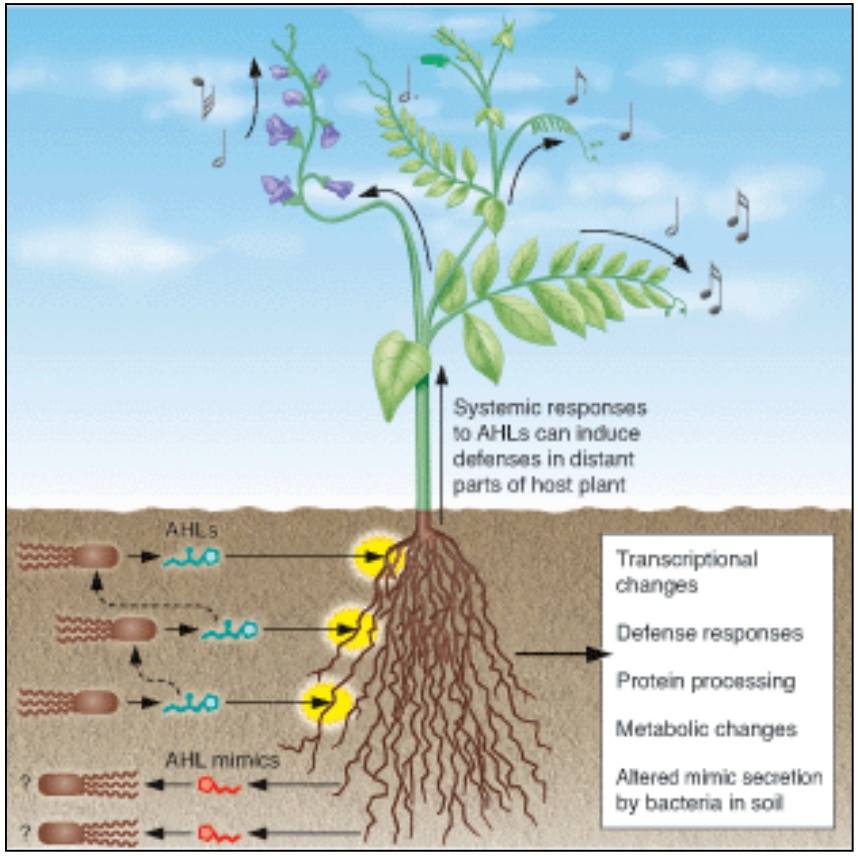
1. escape the immune response of the host,
2. to be able to establish a successful infection.

Manipulation of QS

Plant-based compounds

Inter-kingdom signalling

- Music to its ears: An eukaryotic host listens to bacterial AHL conversations.
- AHLs produced by associated bacteria are detected by both the host and the bacteria.
- The host responds by altering many functions, including defenses, metabolism and production of AHL mimic compounds.
- Systemic responses to the bacterial AHLs can induce defenses in distant parts of the host.



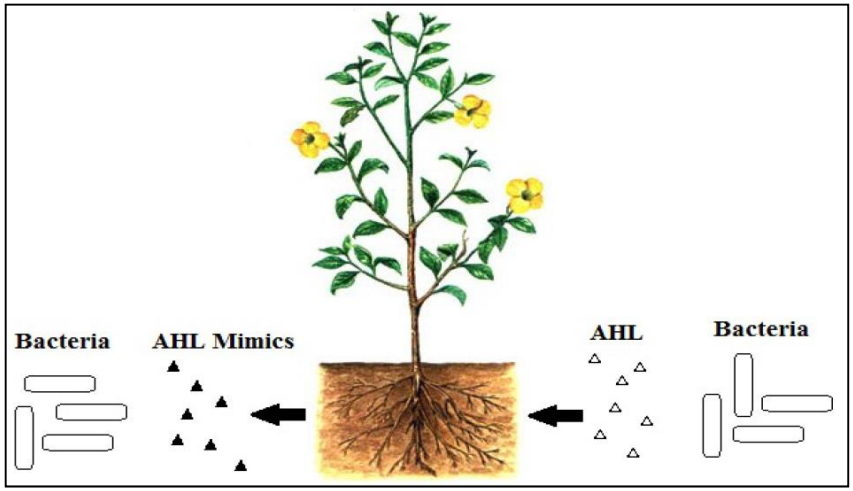
Bauer *et al.*,2005

Manipulation of QS

Plant-based compounds

Plant AHL mimics

- A schematic figure which shows the QS interactions between the plants and the bacteria.
- Bacteria release AHL molecules [△] which can cause virulence such as biofilms and crown gall formation.
- The plants, on the other hand, will response to the bacteria signaling molecules by releasing AHL mimic compounds [▲] for defense purposes and this will then affect the bacterial QS system.



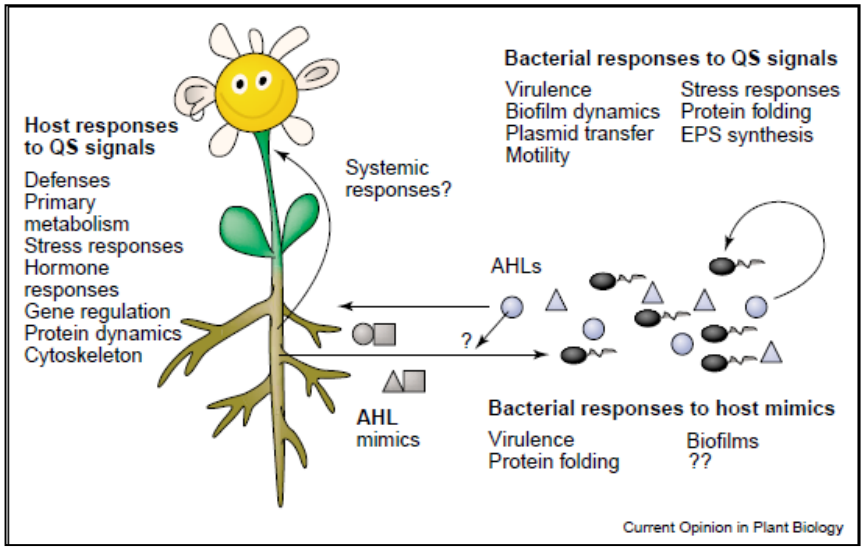
Koh *et al.*,2013

Manipulation of QS

Plant-based compounds

Inter-kingdom signalling

- AHL QS signals (triangles and circles) from bacteria (ovals) affect QS-regulated behaviors in the bacteria and also elicit a diversity of responses in the plant.
- The plant produces and secretes AHL mimic compounds (circle/square, triangle/square) that disrupt or manipulate QS-regulated behaviors in the bacteria.
- Plant responses to bacterial AHLs might affect the secretion of AHL mimic compounds.
- AHL mimics from the plant may also affect synthesis of AHLs in the bacteria.

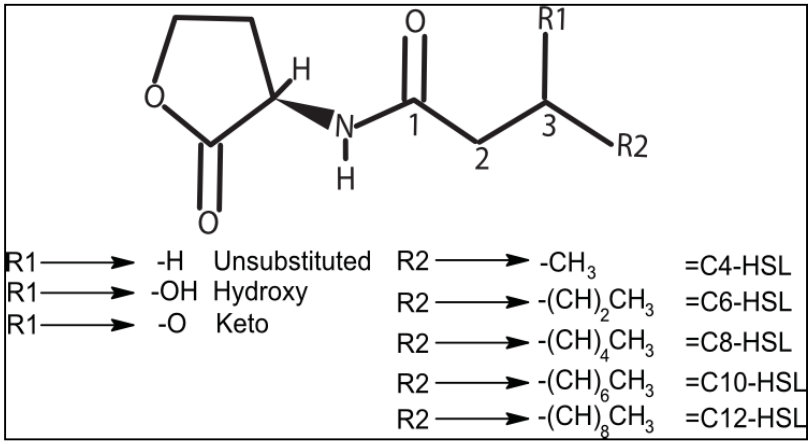


AHL signals in Gram-ve bacteria

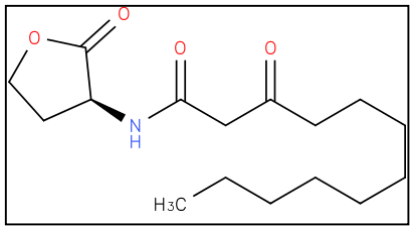
Structure of Acyl homoserine lactones (AHLs)

1. R1 group is defined as substitutions on the third carbon, and
2. R2 group is defined as acyl chain length.

- The acyl chains can vary with even carbon numbers starting from C4, C6, C8 to up to 12 carbons or even more.
- Odd number of carbons in the side chains has also been reported.



Usual substitutions are on the third position (R1 in Figure 3) with hydrogen (un-substituted), a keto (3-oxo), or a hydroxy (3-OH).



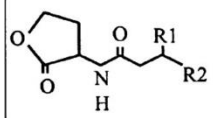
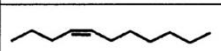
N-(3-oxododecanoyl)-L-homoserine lactone.

Annous *et al.*,2009

AHL signals in Gram-ve bacteria

Structure and functions of AHLs

- Acyl side-chain length and the substitutions on the side chain provide signal specificity.
- Acyl side chains of the signals can be fully saturated, they can have hydroxyls(OH) or carbonyls(C=O) on the third carbon, and they can have lengths of 4 to 16 carbons.
- R_1= –H, –OH or =O;
- R_2= $–CH_3$, $–(CH_2)_{2–14}CH_3$ or $–(CH_2)_5CH=CH(CH_2)_5CH_3$.

Structure	R		AHL molecule	Bacteria	Behaviour
	R1	R2			
 The general structure of AHLs (acyl-HSLs)	H	H	C4-HSL	*S. liquefaciens*	Cell motility/swarming
	OH	H	3-hydroxy-C4-HSL	*V. harveyi*	Bioluminescence
	H		C6-HSL	*C. violaceum*	Pigments/antibiotics/chitinase
	O		3-oxo-C6-HSL	*E. carotovora* *P. aureofaciens*	Pathogenicity/antibiotics Biocontrol activity/antibiotics
	H		C7-HSL	*R. leguminosarum*	?
	O		3-oxo-C8-HSL	*A. tumefaciens*	Conjugation
	OH		3-hydroxy-C14:1-HSL	*R. leguminosarum*	Growth inhibition/rhizosphere genes

Fray,2001;..

Anti-Quorum sensing
Quorum quenching (QQ)

1. You can either make them deaf or
2. you can make them mute (Bassler, the pioneer in quorum-sensing research).

- She is hopeful that the start-up will find new antibacterials.
- This knowledge may lead to new approaches to management either by:

1. Preventing activities of harmful bacteria, or
2. Facilitating activities of helpful ones.

Quorum-quenching (QQ)

Influence of bacteria/eukaryotes on bacterial communication

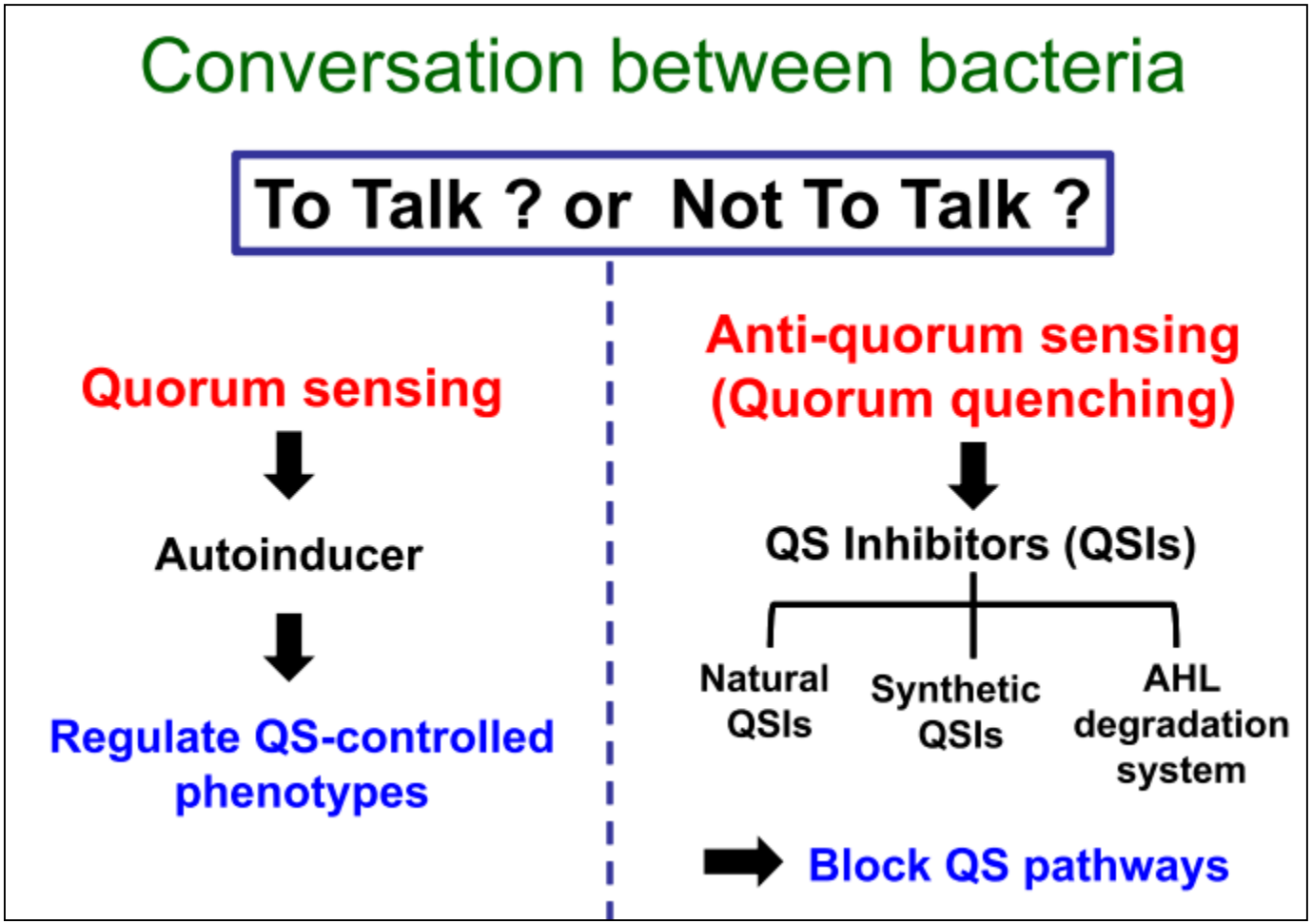
- QQ properties (chemicals and enzymes) are naturally found in various living organisms, like:

1. Bacteria (e.g. *Rhodococcus*, *Commamonas*, etc.),
2. Plants (carrot, soybean, pea seedling, chilli, garlic etc), and
3. Animals (human sera, pork kidney tissues).

Elmerich *et al.*,2008

Quorum-quenching (QQ)

Influence of bacteria/eukaryotes on bacterial communication



Haung,2011

Quorum quenching (QQ)

Quorum-quenching (QQ) bacteria

Anti-QS in the rhizosphere

- QQ bacteria can be divided into three phyla, including:

1. Firmicutes, such as *Bacillus* sp., *Geobacillus* sp. and *S. silvestris*.
2. Actinobacteria, such as *Arthrobacter*, *Microbacterium. testaceum*, *Rhodococcus erythropolis*, *M. avium* and *Streptomyces*.
3. Proteobacteria, such as *Agrobacterium*, *V. paradoxus*, *R. solanacearum*, *Shewanella* sp., *P. aeruginosa*, *Comamonas* sp., *Burkholderia* sp., and *Acinetobacter* sp..

- Most of the demonstrated genera that have the ability to quench QS enzymatically are:
- *Agrobacterium*, *Ochrobactrum*, *Variovorax*, *Comamonas*, *Ralstonia*, *Delftia*, *Burkholderia*, *Pseudomonas*, *Acinetobacter* and *Shewanella*.

Chen *et al.*,2013

Quorum quenching

The anti-Pseudomonas quorum-sensing molecules

A promising approach to prevent bacterium to become pathogenic

- A quorum sensing inhibitor and the structure of the inhibitor bound to the quorum-sensing receptor.
- We developed molecules that inhibit *P. aeruginosa* quorum sensing and these molecules are effective at saving animals and human tissue culture cells from killing by this pathogen.

Bassler Lab.

Quorum quenching (QQ)

Quorum quenching (QQ) bacteria

Bacterial anti-QS in the rhizosphere of tobacco

- In studying the ecosystem of the tobacco rhizosphere, Uroz *et al.*,2003 isolated 25 strains responsible for the degradation of the QS signal molecule AHLs.
- The representative strains of the isolates were identified as members of the genera:
- *Pseudomonas*,
- *Comamonas*,
- *Variovorax*, and
- *Rhodococcus*.

Chen *et al.*,2013

Quorum quenching (QQ)

QQ bacteria

Anti-QS in the rhizosphere of potato

Strains	*rrs* sequencing identification	GenBank Acc. no.[a]	Gram	Colony and cell morphology	Degradation ability[b]				
					C6-HSL	C8-HSL	C12-HSL	C14-HSL	EMPCC NAHL[c]
S6	*Bacillus* sp.	EU977693.1	+	White, rod shape, motile	++	++	++	++	++
S22	*Bacillus* sp.	HM748447.1	+	White, rod shape, motile	++	++	++	++	++
S27	*Bacillus* sp.	HM776218.1	+	White, rod shape, motile	++	++	++	++	++
AM35	*Bacillus* sp.	HM748447.1	+	White, rod shape, motile	++	++	++	++	++
AM38	*Bacillus* sp.	EU240440.1	+	White, rod shape, motile	++	++	++	++	++
EA60	*Bacillus* sp.	FJ866758.1	+	White, rod shape, motile	++	++	++	++	++
EA73	*Bacillus* sp.	AY948211.1	+	White, rod shape, motile	++	++	++	++	++
EA85	*Bacillus* sp.	D26185.1	+	White, rod shape, motile	++	++	++	++	++
DMS133	*Bacillus* sp.	HM188452.1	+	White, rod shape, motile	++	++	++	++	++
S5	*Mesorhizobium*	AF410896.1	–	White, rod shape, motile	++	++	++	++	++
EA113	*Streptomyces* sp.	HM748050.1	+	White, like filamentous, non-motile	+	+	++	++	+
AM51	*Arthrobacter* sp.	AY444858.1	+	White to grayish, rod, non-motile	++	++	+	+	++
AM43	*Arthrobacter* sp.	AY731366.1	+	White to grayish, rod, non-motile	++	++	+	+	++
S15	*Arthrobacter* sp.	AY635865.1	+	White to grayish, rod, non-motile	++	++	+	+	++
EA101	*Pseudomonas* sp.	AJ969084.1	–	White, rod, fluorescent on King-B, motile	++	++	+	+	+
SM88	*E. coli*	–	–	White, rod shape, motile	++	++	++	++	++

Mahmoudi *et al.*,2011

Anti-Quorum sensing

Mode of actions

Biotic factors within the bacterial community

1. AHL inactivation by bacterial enzymes
2. Cross-talk
3. Bacterial AHL mimics
4. Signal interception(stop or seize)
5. Barrier to AHL diffusion.

Boyer and Wisniewski-Dyé,2009

The biocontrol approach

Quorum-quenching (QQ) bacteria

A novel strategy for plant diseases management

- Among the quorum quenching strategies, the potent effect of enzyme based AHL degradation has been identified in a wide diversity of soil bacteria, including:

1. Proteobacteria(Gram-negative bacteria)
2. Low G+C Gram-positive bacteria, and
3. High G+C Gram-positive bacteria.

Park *et al.*,2006

The biocontrol approach
AHL-degrading bacteria

- A mass screen of bacteria isolated from the root system of wild-type plants and plants producing N-AHSL permitted the identification of additional:
- *Bacillus* strains,
- *Agrobacterium* spp.
- *Sphingopyxis witflariensis*
- *Variovorax paradoxus*
- *Bosea thiooxidans*
- *Ralstonia* spp., and
- *Pseudomonas* spp. inactivating N-AHSL.

Faure and Dessaux,2007;…

The biocontrol approach
AHL-degrading bacteria

- Other bacteria, including:
- *Arthrobacter* sp.,
- *Klebsiella pneumoniae*,
- *Variovorax* sp.,
- *Comamonas* sp., and
- *Rhodococcus erythropolis*
- All harbor enzymes capable of AHL destruction.
- Sometimes the genes required for signal generation (*luxI* homologs) and response (*luxR* homologs) are not found on the bacterial chromosome but on plasmids:
- *Agrobacterium* Ti plasmid, and
- *Rhizobium* symbiotic plasmids.

Rasmussen *et al.*,2005;West *et al.*,2007

The biocontrol approach
AHL-degrading bacteria

- *Streptomyces* species were also targeted as sources of diverse quorum-quenching agents, since these bacteria possess the ability to:

1. Synthesize and secrete a variety of secondary metabolites, as well as
2. Various extracellular hydrolytic enzymes.

- Interestingly, a *Streptomyces* sp. was found to interfere with quorum sensing by means of an extracellular AHL-degrading enzyme rather than an AHL competitor.

Park *et al.*,2005

Mechanisms of QS inhibition
Mushroom bacterial pathogens

- Strains of *Burkolderia gladioli* pv. *agariciola*, *Pseudomonas agarici* and *Pseudomonas gingeri*, **but not** those of *Pseudomonas tolaasii* and *Pseudomonas reactans*, produced an array of AHLs depending on the strain.
- This is the first report of AHL production by mushroom bacterial pathogens.
- In the culture filtrates of a certain number of isolates/strains the AHL-hydrolyzed forms were also present.

Shanmugam *et al.*,2011

Mechanisms of QS inhibition
Mushroom bacterial pathogens

- Bioreporter induction by *Agaricus bisporus*, *Pleurotus ostreatus* and *Pleurotus eryngii* cultivated mushroom bacterial pathogens (*Burkolderia gladioli* pv. *agariciola*, *Pseudomonas agarici* and *Pseudomonas gingeri*) as determined in the T-Streak bioassay.

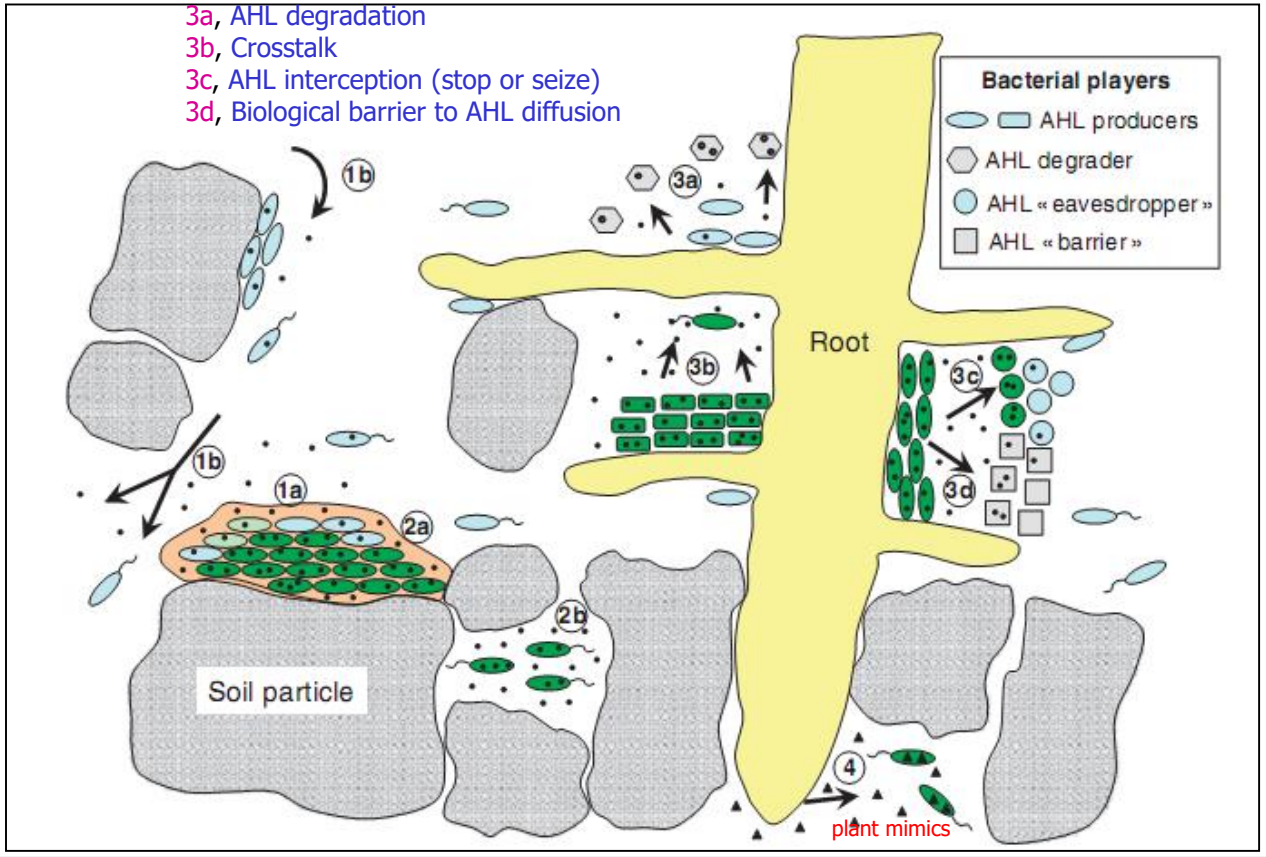
	bioreporters[a]			
bacteria	*Chromobacterium violaceum* (CV026)	*Escherichia coli* (PSB 401)	*Escherichia coli* (PSB 1075)	*Agrobacterium tumefaciens* (pDCI41E33)
Pseudomonas tolaasii	0/20	0/20	0/20	0/20
Pseudomonas reactans	0/20	0/20	0/20	0/20
Pseudomonas gingeri	1/1	0/1	0/1	0/1
Pseudomonas agarici	0/5	3/5	0/5	3/5
Burkholderia gladioli pv. *agaricicola*	4/4	0/4	0/4	0/4

[a] The numerator represents the positive strains, whereas the denominator represents the total number of strains used in the bioassays.

Shanmugam *et al.*,2011

How bacteria communicate among their own kind and with other species

Schematic representation of environmental factors affecting the synthesis, stability, diffusion and perception of AHL signals

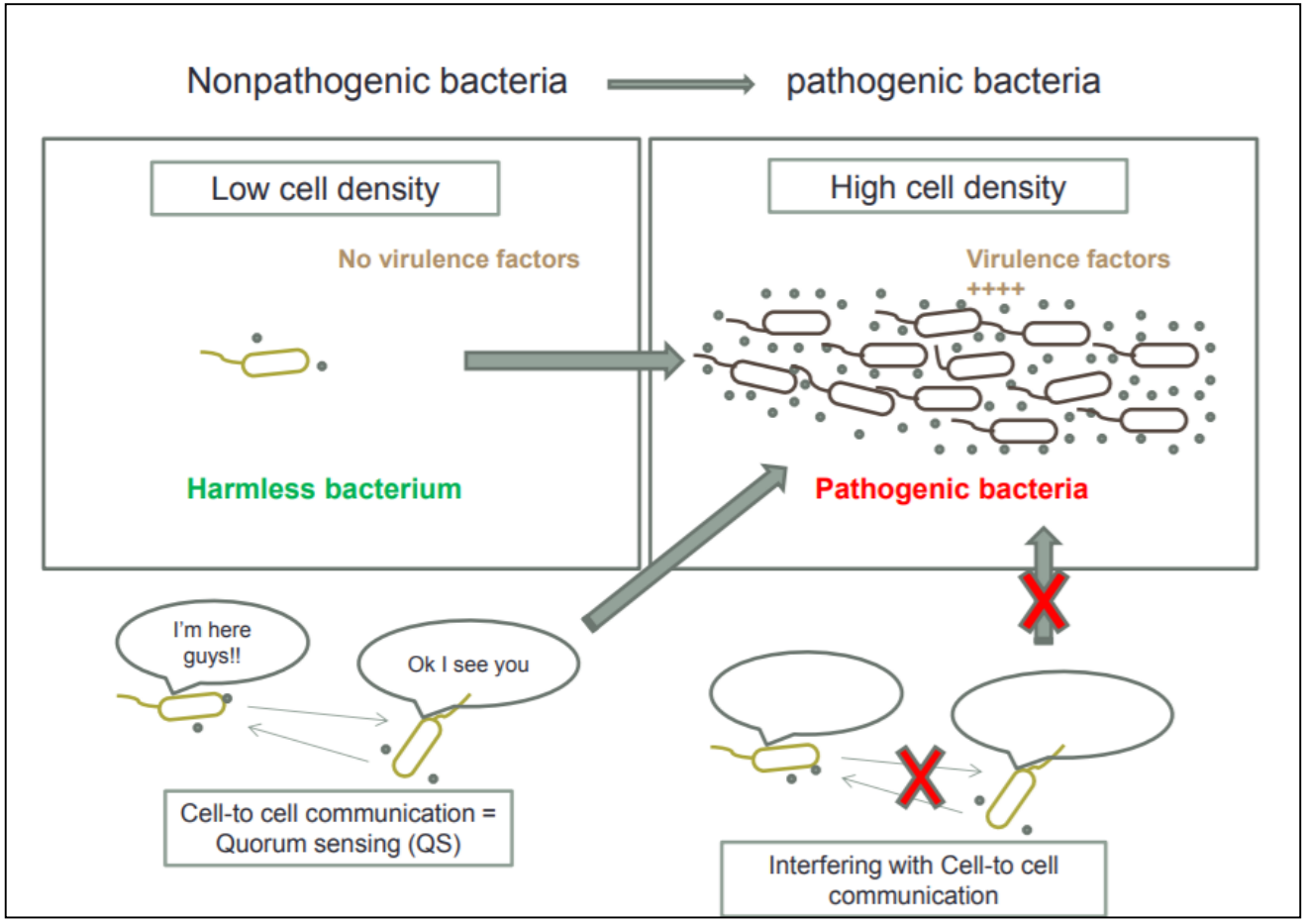


AHL (small dark circles) and plant mimics (small dark triangles) are represented. Light blue cells do not 'quorate' (not having a quorum) whereas green cells 'quorate'.

Boyer and Wisniewski-Dyé,2009

Quorum quenching

A promising approach to prevent bacterium to become pathogenic



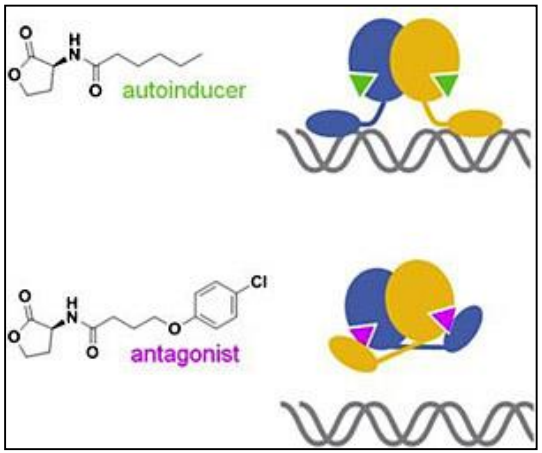
The biocontrol approach

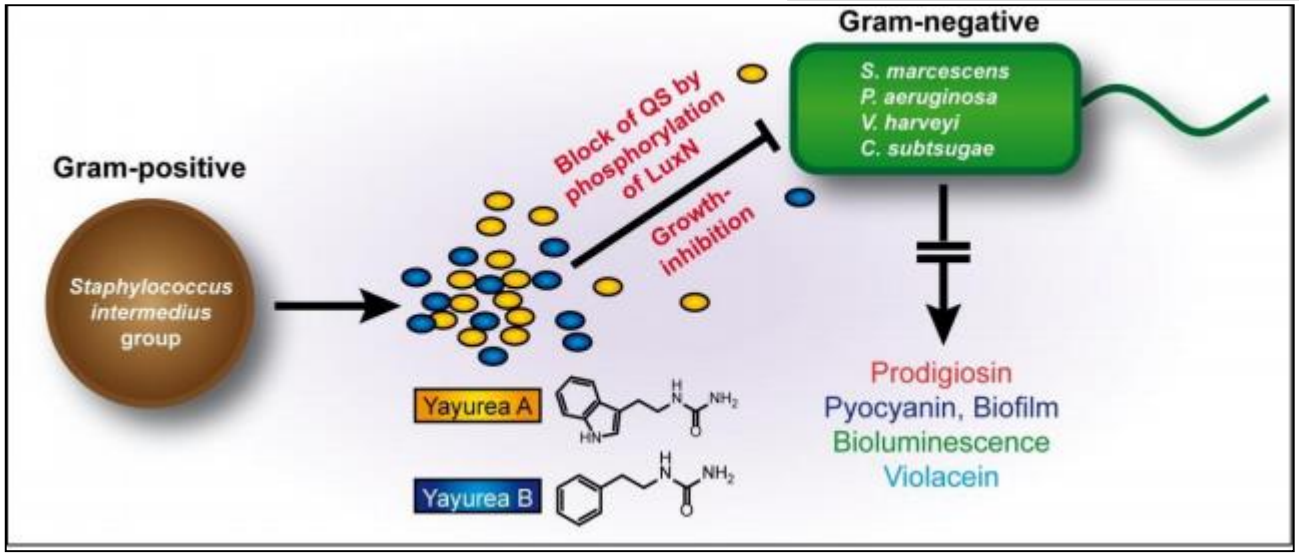
Quorum-quenching (QQ)

A novel strategy for diseases management

Co-existence mechanism of gram-positive staphylococci and gram-negative bacteria.

The staphylococci secrete two compounds (Yayurea A and B) that inhibit the growth and chemical communication (quorum sensing, QS) of the Gram-negative bacteria.

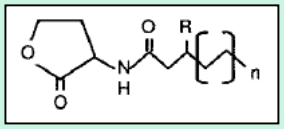
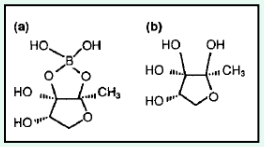
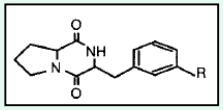
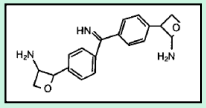
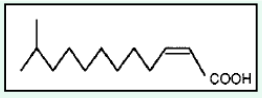




Autoinducers

Gram-negative bacteria

Six types of autoinducers

Autoinducer(s)	General structure	Producing species	Phenotype(s) regulated
AHL(AI-1)		Many Gram-ve bacteria	Motility, exopolysaccharides, biofilms, others
AI-2 (LuxS)		Both Gram-ve and Gram+ve bacteria	Bioluminescence, ABC transporters
AI-3		Some species of Gram-negative bacteria	responsible for activating gene expression in *Salmonella*
Cyclic dipeptides		*P. fluorescens*, *P. alkaligenes*	Cross activate quorum sensing indicator strains
Bradyoxetin		*B. japonicum*	Nodulation
DSF		*X. campestris*	Endoglucanase production

González and Keshavan,2006;..

Mechanisms of QS inhibition

A. Bacterial-based compounds

AA. AHL-degrading or modifying enzymes

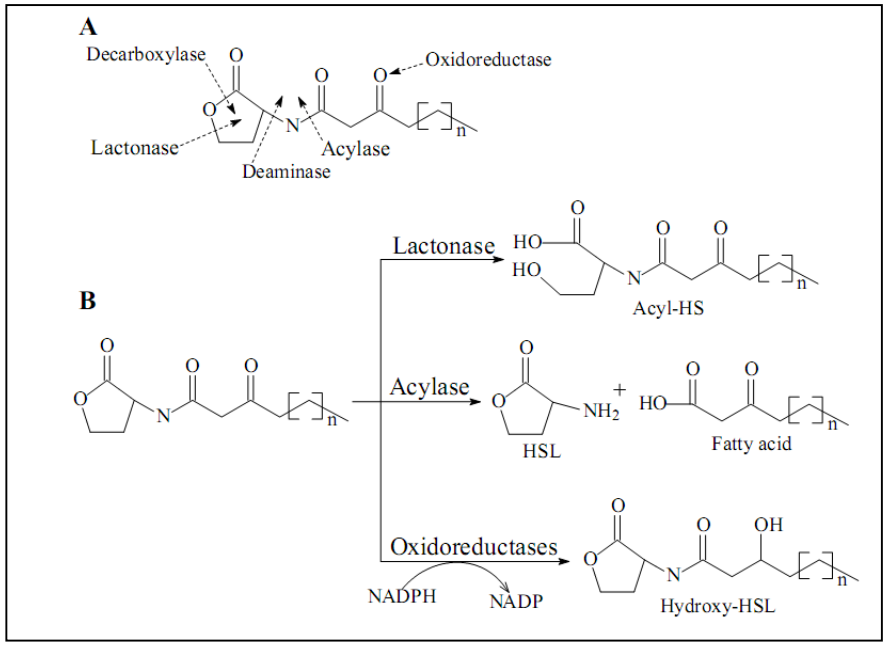
- Quorum quenching enzymes have been identified in quorum sensing and non-quorum sensing microbes, including:

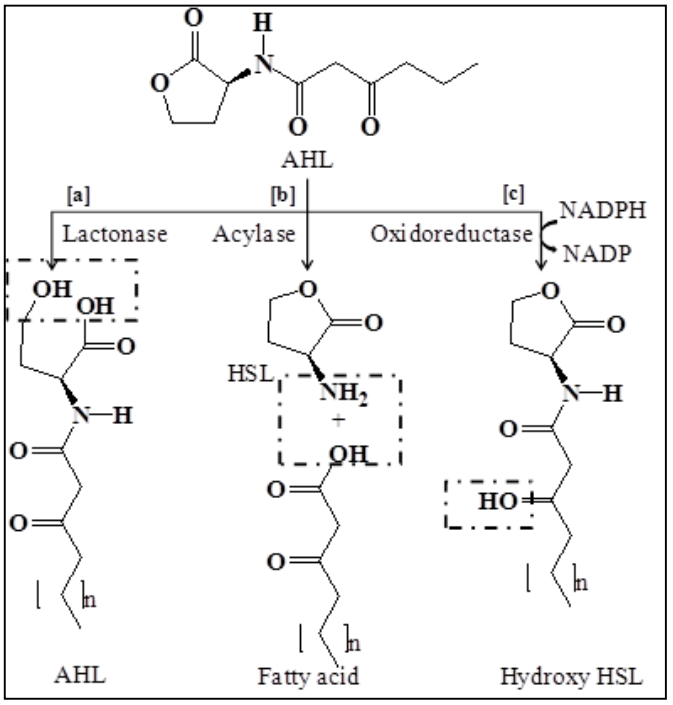
1. Lactonase (inactivates AHLs by hydrolyzing the lactone bond to produce corresponding *N*-acyl homoserines),
2. Acylase (cleaves the amide bond (-CONH2) of AHL by releasing fatty acid),
3. Oxidoreductase (modifying enzyme-catalyze the oxidation or reduction of acyl side chain), and
4. Paraoxonase (degrades AHL by hydrolyzing its lactone ring).

Chen *et al.*,2013;..

Mechanisms of QS inhibition

AHL-degrading or modifying enzymes





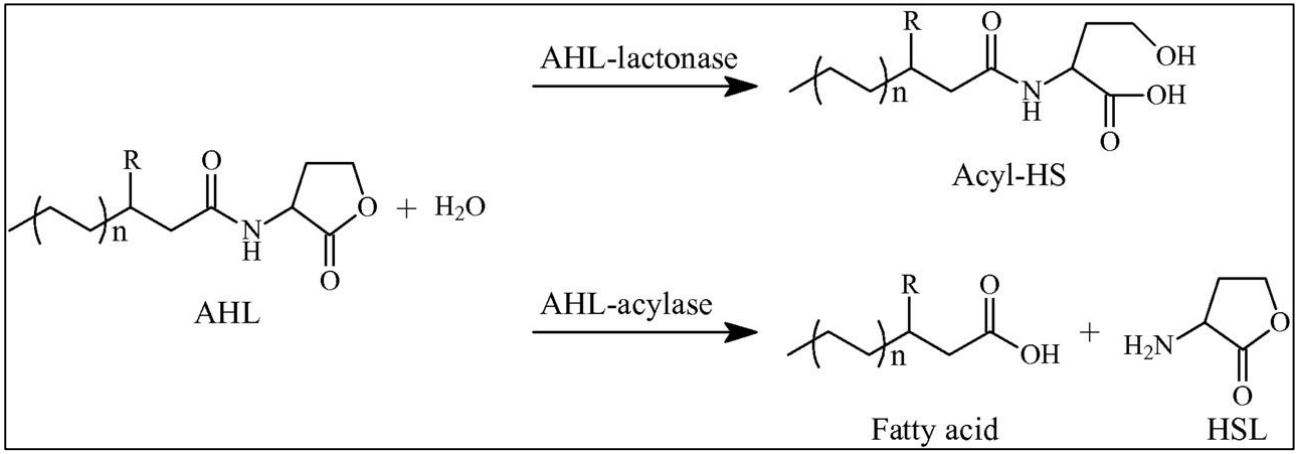
Broken lines mark position of possible cleavages by following enzymes: 1, lactonase; 2, decarboxylase; 3, acylase; 4, deaminase.

Dong & Zhang,2005; Chen *et al.*,2013; Lade *et al.*,2014

Mechanisms of QS inhibition

Two main types of AHL-degrading enzymes

1. AHL lactonases hydrolyze the lactone ring in the homoserine moiety of AHLs, without affecting the rest of the signal molecule structure.
2. AHL-acylases break the amide linkages of the AHLs (amidohydrolase).

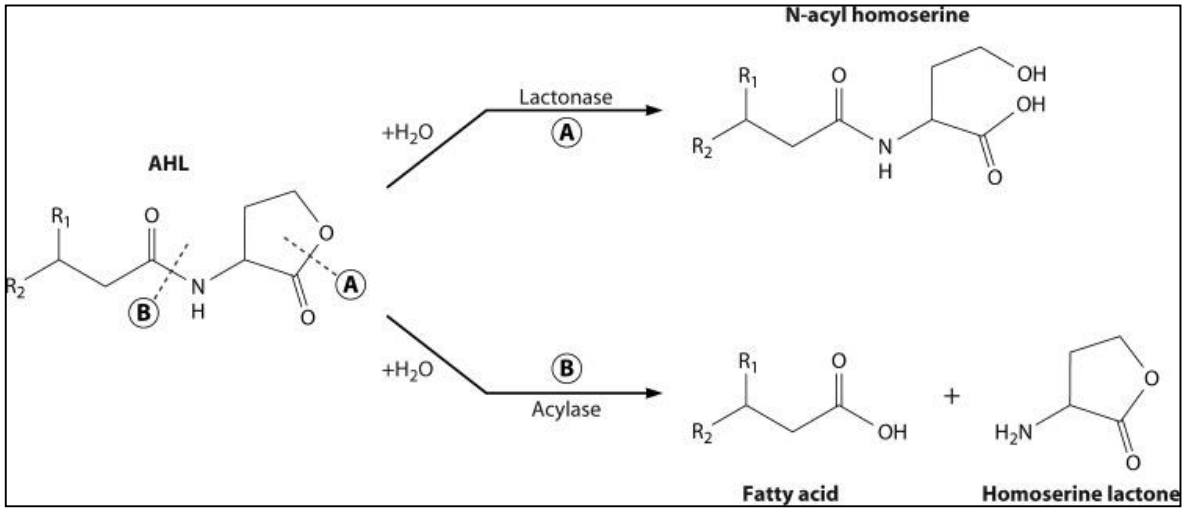


Wang *et al.*,2004

Mechanisms of QS inhibition

Two main types of AHL-degrading enzymes

- AHL acylases hydrolyze the acyl-amide bond between the acyl tail and latone ring of AHLs in a nonreversible manner, resulting in the release of a fatty acid chain and a homoserine lactone moiety.



LaSarre *et al.*,2013

Mechanisms of QS inhibition

Bacterial enzymes as quorum sensing inhibitors

Source of quorum sensing inhibitor	Enzyme	Degraded quorum sensing signal
Bacillus sp.strain 240B1	Lactonase	AHLs
Bacillus thuringiensis	Lactonase	AHLs
Stappia[a] sp. strains 5, 176 and 97-1	Lactonase	AHLs
Oceanobacillus strains 30, 172, and 97-2	Lactonase	AHLs
Halomonas sp. strain 33	Lactonase	AHLs
Tenacibaculum discolor strain 20J	Acylase/Lactonase	AHLs
Hyphamonas sp. DG895	Acylase/Lactonase	C4HSL and 3OC12-HSL
Alteromonas sp. strain 168	Acylase	C4HSL and 3OC12-HSL
Bacillus megaterium	AHL-oxidase	C4HSL and 3OC12HSL
Bacillus circulans strain 24	Different from Lactonase[b]	C4HSL and 3OC12HSL
Bacillus pumilus S8-07	AHL-acylase	3OC12HSL
Ralstonia sp. XJ12B	AHL-acylase	Long chain AHLs
Pseudomonas aeruginosa PAO1	AHL-acylase	Long chain AHLs
Rhodococcus erythropolis strain W2	AHL-Lactonase	AHLs
	Acylase (Amidohydrolase)	AHLs
	Oxidoreductase activity	3-oxo-N-AHLs
Burkholderia strain GG4	AHL – oxidoreductase	3OC6HSL
Agrobacterium tumefaciens	AHL-Lactonase	AHLs
Arthrobacter sp. IBN110	AHL-Lactonase	AHLs
Acinetobacter sp. strain C1010	Lactonase	AHLs

Kalia,2013

Mechanisms of QS inhibition

AHL-degrading or modifying enzymes

1. QQ bacteria with AHL-Lactonase activity

- *Bacillus* species were amongst the first bacteria reported to degrade AHL by producing lactonase enzymes, which inactivate AHLs by opening the lactone ring.
- Broad-spectrum AHL-degrading AiiA enzymes were found to be widespread in the:
- *Bacillus thuringiensis*
- *B. cereus* strains
- *B. amyloliquefaciens*
- *B. subtilis*
- *B. mycoides*, and
- *B. marcorestinctum*.

Chen *et al.*,2013;..

Mechanisms of QS inhibition
QQ bacteria with AHL-Lactonase activity

- *A. tumefaciens* producing AttM and AiiB.
- *Arthrobacter* producing AhlD.
- *Klebsiella pneumonia* producing AhlK.
- *Ochrobactrum* producing AidH.
- *Microbacterium testaceum* producing AiiM.
- *Solibacillus silvestris* producing AhlS.
- *Rhodococcus strains* W2, LS31 and PI33 producing QsdA.
- *Chryseobacterium* strains isolated from the plant root have been shown to degrade AHL, and some strains showed putative AHL-lactonase activity.

Chen *et al.*,2013

Mechanisms of QS inhibition

QQ bacteria with AHL-Lactonase activity

Enzyme	Host	Substrate
	AHL lactonase	
	Bacillus sp. 240B1	C6-10-HSL
	Bacillus cereus A24	AHL
AiiA	*Bacillus mycoides*	AHL
	Bacillus thuringiensis	AHL
	Bacillus anthracis	C6, C8, C10-HSL
AttM	*Agrobacterium tumefaciens*	3-oxo-C8-HSL, C6-HSL
AiiB	*Agrobacterium tumefaciens* C58	Broad
AiiS	*Agrobacterium radiobacter* K84	Broad
AhlD	*Arthrobacter* sp. IBN110	Broad
AhlK	*Klebsiella pneumoniae*	C6-8-HSL
QlcA	*Acidobacteria*	C6-8-HSL
AiiM	*Microbacterium testaceum* StLB037	C6-10-HSL
QsdA	*Rhodococcus erythropolis* W2	C6-14-HSL with or without C3-substitution
AidH	*Ochrobactrum* sp. T63	C4-10-HSL
DlhR, QsdR1	*Rhizobium* sp. NGR234	nd.
AhlS	*Solibacillus silvestris* StLB046	C6-HSL, C10-HSL
SsoPox	*Sulfolobus solfataricus* strain P2	C8-12-HSL
	Rhodococcus sp.	Broad
GKL	*Geobacillus kaustophilus* strain HTA426	C6-12-HSL
PPH	*Mycobacterium tuberculosis*	C4, C8, C10-HSL,
MCP	*Mycobacterium avium* subsp. *paratuberculosis*	C7-12-HSL
BpiB01, BpiB04, BpiB05, BpiB07	Soil metagenome	3-oxo-C8-HSL
QlcA	Soil metagenome	C6-10-HSL

nd, not determined.

Chen *et al.*,2013

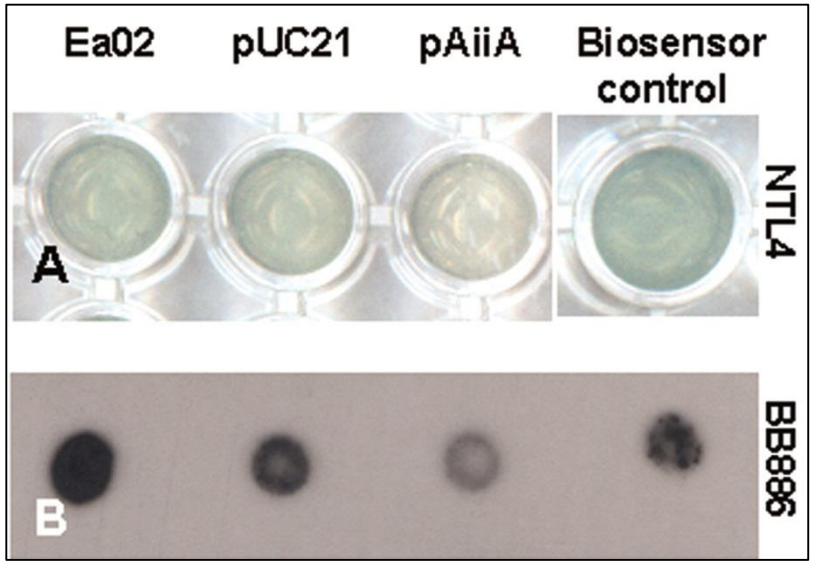
Mechanisms of QS inhibition

QQ bacteria with AHL-Lactonase activity

Manipulation of QS *Erwinia amylovora*

- Autoinducer production by *E. amylovora* and autoinducer degradation in a derivative strain carrying the *aiiA* acyl-homoserine lactonase-encoding gene.
- Heterologous expression of the *Bacillus* sp. strain A24 acyl-homoserine lactonase gene *aiiA* in *E. amylovora:*

1. Abolished induction of AHL biosensors,
2. Impaired extracellular polysaccharide production and tolerance to hydrogen peroxide, and
3. Reduced virulence on apple leaves.



Autoinducer production was revealed by the appearance of a blue color resulting from β-galactosidase activity in the NTL4/pZLR4 AHL biosensor or by an increase in the intensity of black spots visualized after autoradiography to detect light emission by the *Vibrio* autoinducer biosensor strain.

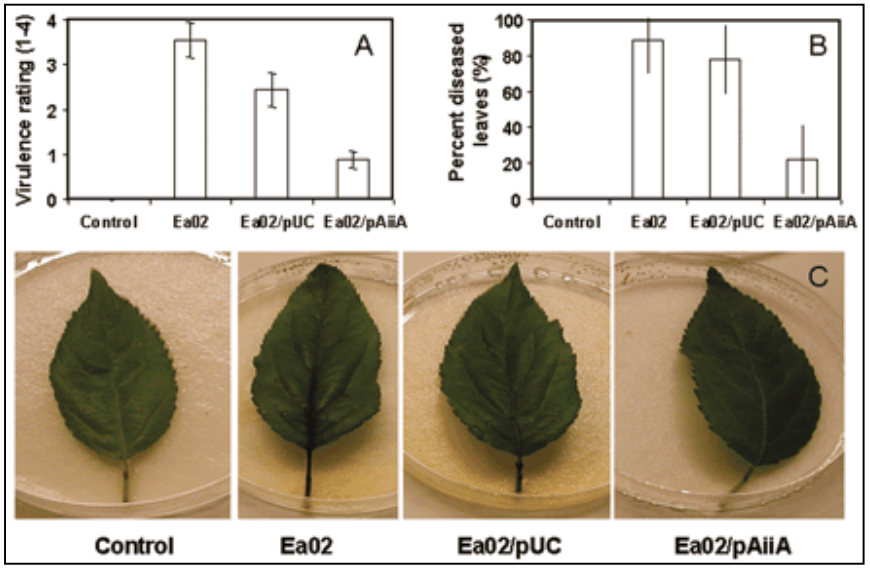
Molina *et al.*,2005

Mechanisms of QS inhibition

QQ bacteria with AHL-Lactonase activity

Manipulation of QS *Erwinia amylovora*

- Virulence attenuation in *E. amylovora* with heterologous expression of the *Bacillus* sp. strain A24 acyl-homoserine lactonase gene, *aiiA*.
- 'Golden Delicious' apple leaves were inoculated with saline (Control), with the wild-type pathogen (Ea02), or with one of its derivatives expressing *aiiA* (Ea02/pAiiA) or carrying the vector plasmid without *aiiA* (Ea02/pUC).
- The virulence severity (A) and disease incidence (B) were determined after 7 days of incubation.



The development of symptoms(leaf vein discoloration and necrosis)was observed in 1) wild-type- and 2) transconjugant control-challenged leaves (C).

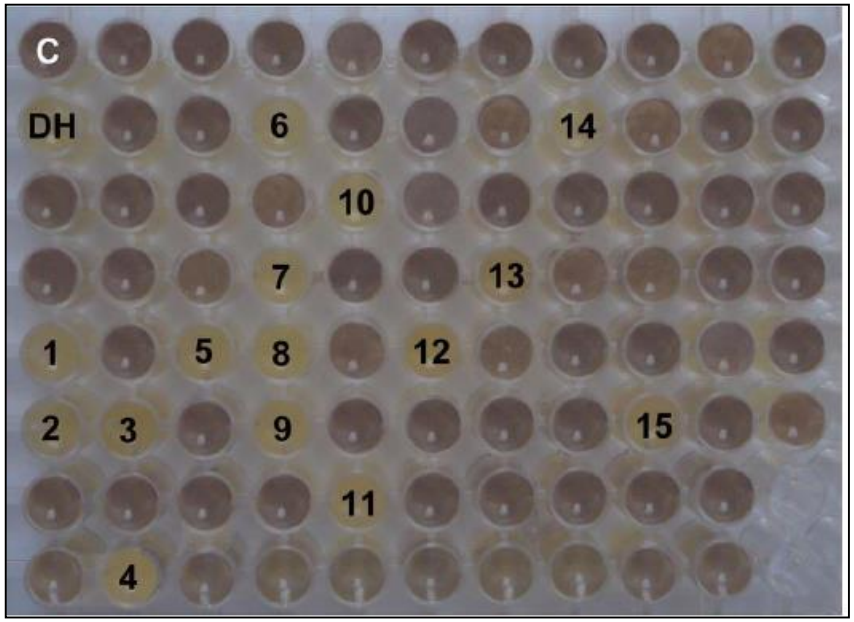
Molina *et al.*,2005

The biocontrol approach

QQ bacteria with AHL-Lactonase activity

Detection of AHL-degrading bacteria

- The NAHL degrading isolates were detected by inhibition of synthesis of violacein by *Chromobacterium violaceum* CV026 in the presence of C6-HSL at 6 mg/l(dissolved in LB).
- The 15 NAHL-degrading isolates are numbered from 1 to 15.
- C (control) degradation assay performed without bacteria.
- The DH is *E. coli* DH5 a (SM88) carrying *aiiA* lactonase encoding gene was used as positive NAHL degrading bacterium.



The picture was taken after 24 h incubation.

Olivero *et al.*,2011 used DMSO as a solvent for dissolving C6-HSL.

Mahmoudi *et al.*,2011

Experimental procedures
Biocontrol activity of bacterial consortia
Soft rot tuber assays

- Maceration symptoms were encoded as follows:

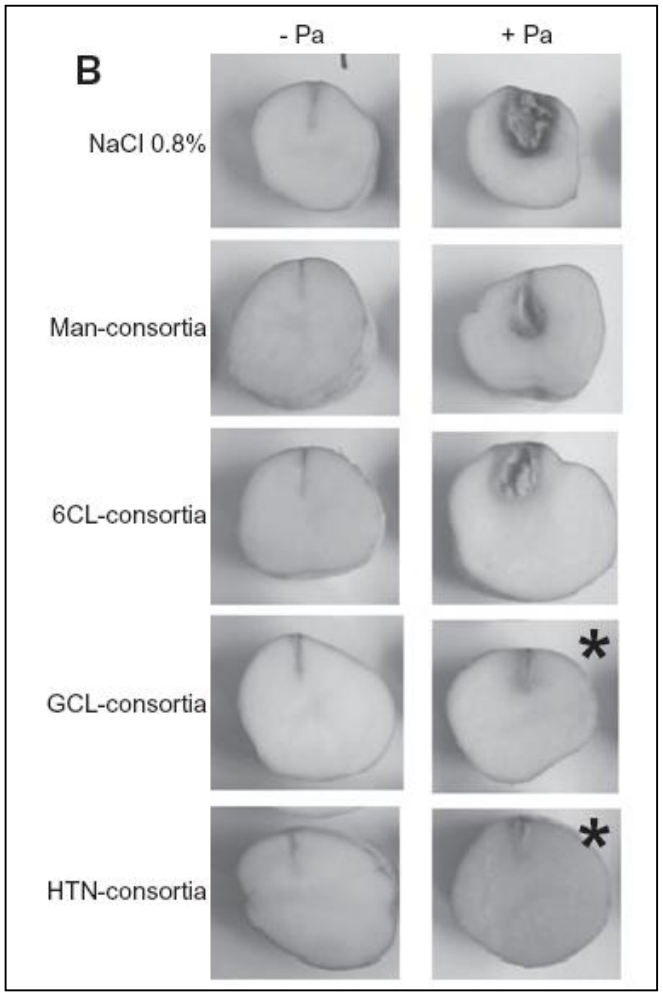
1. No maceration;
2. Moderate maceration (no more than 5 mm around the infection site);
3. Strong maceration (more than 5 mm).

Difference between a consortium and a mixed culture:
A microbial consortium or consortium, is two or more bacterial or microbial groups (several taxa/genera) living symbiotically. Consortiums can be endosymbiotic or ectosymbiotic. A true microbial consortium is a group of two or more different species that work together and function at a higher level than they could alone. Whereas, a mixed culture is group of microbes have either different or same functions for one or other objective.

Experimental procedures
Biocontrol activity of bacteria consortia
Soft rot tuber assays

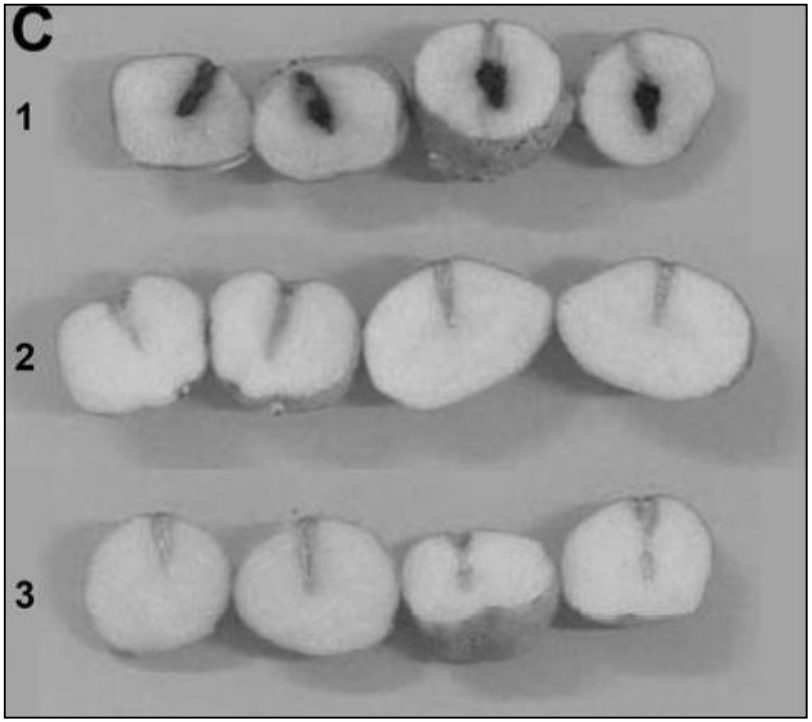
- *Pectobacterium atrosepticum* cells, as well as bacterial consortia from mannitol-(control), 6CL-, GCL- and HTN-enrichments, were washed in NaCl 0.8%.
- In each tuber of *S. tuberosum* var. allians were inoculated by c. $2x10^7$cfu of *P. atrosepticum* and/or $2x10^6$ cfu of each of bacterial consortia.
- Eighteen tubers were inoculated for each condition in two independent experiments.
- Asterisk indicates a statistically significant decrease of the maceration symptoms.

Cirou *et al.*,2007



Experimental procedures
Biocontrol activity of bacterial consortia
Soft rot tuber assays

- Visual observation of potato tuber maceration induced by:
1. *Eca* 6276(pME6000);
2. *Eca* 6276(pME6863);
3. The sterile control consisting in 0.9% NaCl
- Pictures were taken 14 days after inoculation.



Smadja *et al.*,2004

Experimental procedures

Biocontrol activity of AHL degrading rhizobacteria against *Pectobacterium atrosepticum*

Potato tuber and greenhouse assays

A. Negative control consisting of a tuber treated with 0.8% NaCl;

B. Inoculation of 20 µl of *P. atrosepticum* strain SM1 alone at about 10^6 CFU per tuber;

C. Co-inoculation of Pa-SM1 at about 10^6 CFU per tuber along EM10 (*Chryseobacterium* sp.).

D. Inhibition of *P. atrosepticum* SM1 pathogenicity in greenhouse by AHL degrading rhizobacterium *Chryseobacterium* sp. EM10.

Mahmoudi *et al.*,2011

PCR amplification of *aiiA* homologue gene

AHL-lactonase activity

- The bacteria which were positive for AHLs degradation bioassay were confirmed for AHL-lactonase activity by amplification of *aiiA* homologue gene.
- Briefly, genomic DNA was amplified using forward and reverse primer aiiAF2 (5'CGGAATTCATGACAGTAAAGAAGCTTTA-3') and aiiAR2 (5'-CGCTCGAGTATATATTCAGGGAACACTT-3').
- The thermal cycling conditions were maintained as initial denaturation at 94°C for 5 min, 5 cycles of 94°C (45s), 44°C (45 s), 72°C (1 min); 30 cycles of 94°C (45 s), 53°C (45 s), 72°C (1 min), followed by primer extension at 72°C for 8 min.
- The PCR amplicons were gel purified and sequenced.
- Sequence obtained was analyzed and the open reading frame (ORF) was detected using Geneious 6.1.

Rajesh and Rai,2013

SDS-PAGE analysis of AHLs

Purification of AHL-lactonase and its variants *Aiia* produced by *B. subtilis*

- The *aiiA* gene encoding AHL-lactonase and its variants H106S, D108S, H109S, and H169S contained in the pGEM-7Zf(+) vector were amplified, respectively, by PCR using forward primer 5′-ATCGGATCCATGACAGTAAAGAAGCTTTATTTCG-3′ and reverse primer 5′-GTCGAATTCCTCAACAAGATACTCCTAATGATGT-3′.
- The PCR products were digested by BamHI and EcoRI and fused in-frame to the glutathione *S*-transferase (GST) gene under the control of the isopropyl β-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter in GST fusion vector pGEX-2T.
- The constructs were verified by DNA sequencing.

Wang *et al.*,2004

SDS-PAGE analysis of AHLs

Purification and properties of AHL-lactonase *Aiia* produced by *B. subtilis*

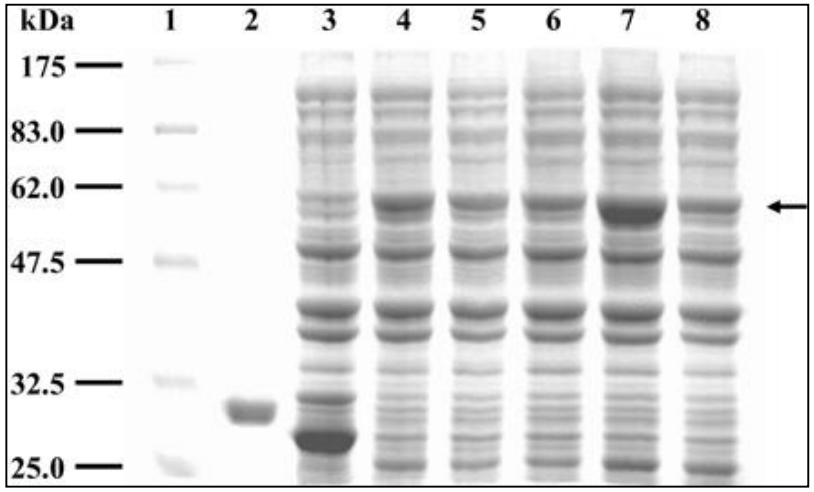
- The GST-AiiA fusion protein was expressed in *E. coli* following IPTG induction and purified by routine GST affinity chromatography procedure.
- The recombinant AiiA (AHL-lactonase), which has two extra amino acid residues (Gly and Ser) at the N terminus than does the native AiiA, was separated from GST by thrombin digestion.
- The recombinant enzyme was purified 86-fold with a yield of ~7.3% of the total proteins.
- The SDS-PAGE analysis indicates that the purity of the obtained recombinant AHL-lactonase (7 μg loaded) should be >98.5%, because staining with Coomassie Brilliant Blue R-250, which can detect as little as 0.1 μg of protein, did not reveal other protein bands.
- The SDS-PAGE analysis showed the size of the purified AHL-lactonase enzyme is ~28 kDa, which is consistent with the predicted molecular mass of 28,036 Da.

Wang *et al.*,2004

SDS-PAGE analysis of AHLs

Aiia produced by *B. subtilis*

- Lane 1, standard molecular mass marker proteins;
- Lane 2, purified AHL-lactonase (7 μg);
- Lanes 3-8, crude cell extracts of *E. coli* containing, respectively, construct pGEX-2T, pGST-AiiA, pGST-H106S, pGST-H169S, pGST-D108S, and pGST-H109S.
- The protein samples were fractionated by 10% SDS-PAGE gel, following by staining with Coomassie Brilliant Blue R-250.



Arrow indicates the location of GST-AiiA and variants.

Wang *et al.*,2004

Mechanisms of QS inhibition

AHL-degrading or modifying enzymes

2. QQ bacteria with AHL-acylase activity

- The AHL-acylase was first described in the *Variovorax paradoxus* strain VAI-C.
- Subsequently, AHL-acylases from various groups of bacteria have been reported, predominantly including

1. AiiD in *Ralstonia* sp XJ12B;
2. AhlM in *Streptomyces* sp. M664;
3. PvdQ and QuiP in *P. aeruginosa* PAO1 and
4. *AiiC* in *Anabaena* sp. PCC7120.

- The acylase AiiD was also reported from *Pseudomonas* spp.
- *R. solancearum* GMI1000 produced a putative aculeacin A acylase with distinct QQ activity.
- *Ralstonia* strain XJ12B can degrade and grow rapidly on short- and long-chain AHLs.

Chen *et al.*,2013

Mechanisms of QS inhibition

QQ bacteria with AHL-acylase activity

AHL acylase		
AiiD	*Ralstonia eutropha*	C8-12-HSL
PvdQ	*Pseudomonas aeruginosa*	C7-12-HSL with or without C3-substitution
QuiP	*Pseudomonas aeruginosa*	C7-14-HSL with or without C3-substitution
AiiC	*Anabaena* sp. PCC 7120	Chain length more than C10
AhlM	*Streptomyces* sp. M664	Chain length more than C8
Aac	*Ralstonia solanacearum*	Chain length more than C6
	Shewanella sp. MIB015	Broad but prefer long chain
HacA	*Pseudomonas syringae*	C8,C10, C12-HSL
HacB	*Pseudomonas syringae*	C6-12-HSL with or without C3-substitution
	Variovorax sp.	Broad
	Variovorax paradoxus	Broad
	Tenacibaculum maritimum	C10-HSL
	Comomonas sp. D1	C4-16-AHL with or without C3-substitution
	Rhodococcus erythropolis W2	C10-HSL

Chen *et al.*,2013

Mechanisms of QS inhibition

QQ bacteria with AHL-acylase activity

Manipulation of QS *P. carotovorum*

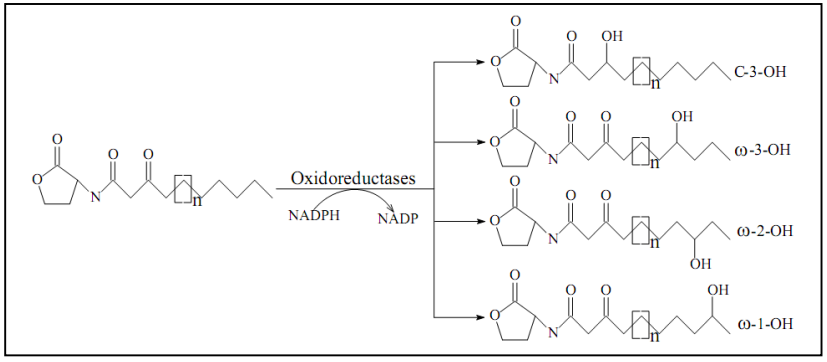
- AHL-acylase AiiD inactivates the OHL of *Pectobacterium carotovorum*.
- AHL-acylase AiiD from *Ralstonia* sp. are known to degrade AHLs (Quorum-Sensing Inhibitors (QSI).
- Some strains of *B. subtilis* destroy the signal molecules of other systems in the rhizosphere and thus disturb the rhizosphere equilibrium.

Mechanisms of QS inhibition

AHL-degrading or modifying enzymes

3. QQ bacteria with AHL-oxidoreductase activity

- AHL oxidoreductase, a novel type of AHL inactivating enzyme found in *B. megaterium* CYP102A1.
- The activity of these enzymes results in silencing the QS-regulated processes, as degradation products cannot act as signal molecules.
- Oxidoreductase targets the acyl side chain by oxidative or reducing activities and thus catalyzes a **modification of the chemical structure of the signal but not degradation**.



Czajkowski and Jafra,2009; Chen *et al.*,2013

Mechanisms of QS inhibition
B. DSF disrupting/degrading bacteria

- Non-AHL signal such as diffusible signal factor (DSF) regulate virulence in several *Xanthomonas* species as well as *Xylella fastidiosa*.

1. A variety of bacteria could disrupt DSF-mediated induction of virulence factors in *Xanthomonas campestris* pv.*campestris*.
2. At the same time, several other bacterial strains belonging to genera:

- *Bacillus*,
- *Paenibacillus*,
- *Microbacterium*,
- *Staphylococcus*, and
- *Pseudomonas* were identified that were capable of particularly rapid degradation of DSF.

Newman *et al*.,2007

Mechanisms of QS inhibition

Introduction

DSF producing bacteria

- DSF-based quorum sensing is a new language in Gram-negative bacteria.
- Diffusible signal factor (DSF) is a fatty acid signal molecule involved in regulation of virulence in several *Xanthomonas* species as well as *Xylella fastidiosa*.
- It also regulates virulence and biofilm dispersal in *Burkholderia* spp. and *Pseudomonas aeruginosa*.
- BDSF was found from *Burkholderia cenocepacia*.

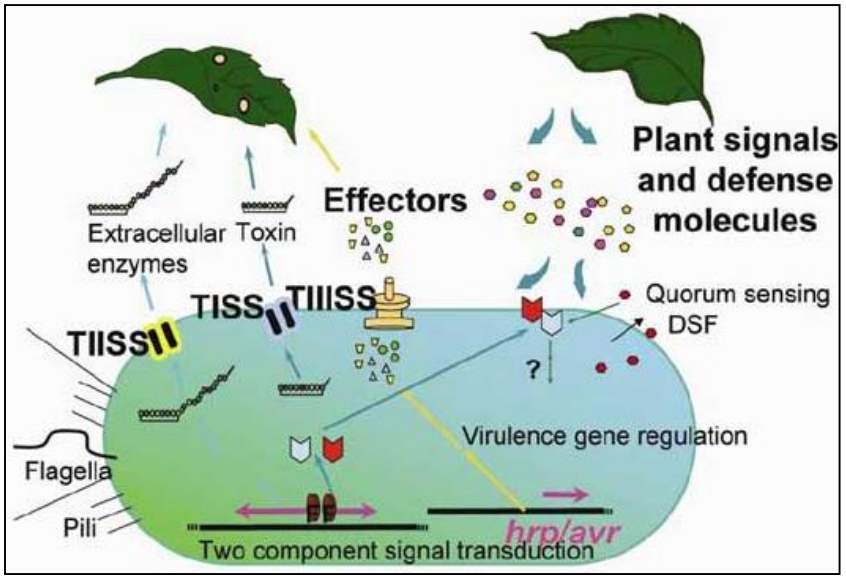
Chatterjee,2009; Deng *et al.*,2010; Wu *et al.*,2011

Mechanisms of QS inhibition

Introduction

DSF producing bacteria

- The most common QS signals in G-ve bacteria are N-acyl homoserine lactones (AHLs).
- But some other non-AHLs (DSF) have been identified in rhizobia and plant pathogens.
- DSF originally identified from the plant bacterial pathogen *Xanthomonas campestris* pv. *campestris*.
- Later, BDSF was found from *Burkholderia cenocepacia*.



Three secretion systems:
TISS,TIISS and TIIISS

Chatterjee,2009; Deng *et al.*,2010; Wu *et al.*,2011

Mechanisms of QS inhibition

Identification of DSF-inhibiting bacteria

- A survey of the 16S ribosomal RNA gene sequence from the DSF-inhibiting isolates revealed that the DSF-inhibiting strains included:

1. Members of the gram-positive genera *Bacillus*, *Paenibacillus*, *Microbacterium*, and *Staphylococcus*.
2. Members of the gram negative genus *Pseudomonas*.

Strain name	Identity[a]	% Identity[b]	Origin	DSF degradation[c]	Mechanism of DSF inhibition
A	*Paenibacillus paduli*	96	Grapevine	+++	Unknown
B	*Paenibacillus paduli*	97	Grapevine	+++	Unknown
C	*Pseudomonas* sp. Bsi20664	99	Cabbage	+++	Enzymatic degradation
D	*Staphylococcus pasteur*	99	Grapevine	+++	Unknown
E	*Bacillus cereus*	99	Broccoli	+++	Enzymatic degradation
G	*Pseudomonas* sp. strainBsi20664	99	Cabbage	+++	Enzymatic degradation
H	*Pseudomonas jessenii Ps06*	99	Cabbage	+++	Enzymatic degradation
J	*Pseudomonas sp. Fa2*	99	Tomato	+++	Enzymatic degradation
L	*Staphylococcus sp. es1*	99	Grapevine	++	Unknown
	Escherichia coli (DH5α)		Lab collection	+	Unknown

Newman *et al.*,2007

Mechanisms of QS inhibition

DSF disrupting/degrading bacteria

Xanthomonas campestris pv. *campestris*

- The plant pathogen *Xanthomonas campestris* pv. *campestris*, expresses tissue-macerating pathogenicity genes upon the accumulation of a diffusible signal factor (DSF).
- This cell-to-cell communication system in which RpfF functions as the DSF synthase is required for virulence.
- DSF from *X. campestris* pv. *campestris* has been characterized as *cis*-11-methyl-2-dodecenoic acid (a fatty acid).
- Mutations in *rpfF* reduce virulence because pathogenicity traits such as cellulases are no longer expressed in *rpfF* mutants.

Newman *et al.*,2007

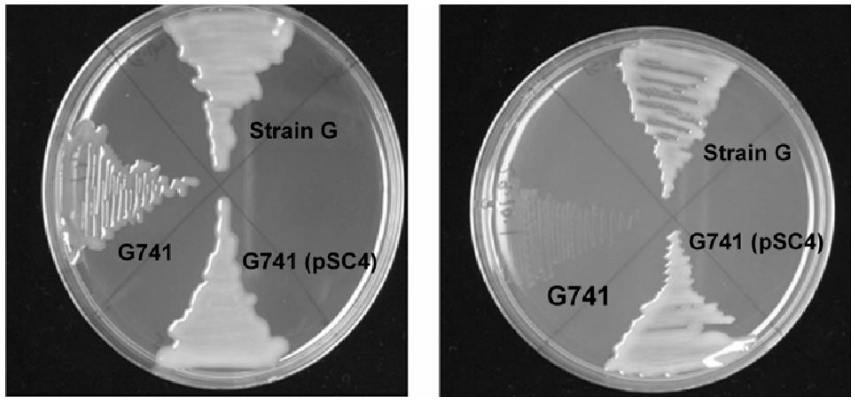
Mechanisms of QS inhibition

DSF disrupting/degrading bacteria

Xanthomonas campestris pv. *campestris*

- Diffusible signal factor (DSF) degradation mutants of Xcc are deficient in exopolysaccharide production in rich King's B (KB) medium.

1. *Pseudomonas* spp. strain G (wild type),
2. DSF degradation-deficient mutant G741, and
3. Strain G741 complemented with plasmid pSC4 were grown for 2 days in rich KB medium and M9 minimal medium.



KB medium (left) and M9 minimal medium (right).

Newman *et al.*,2007

Mechanisms of QS inhibition

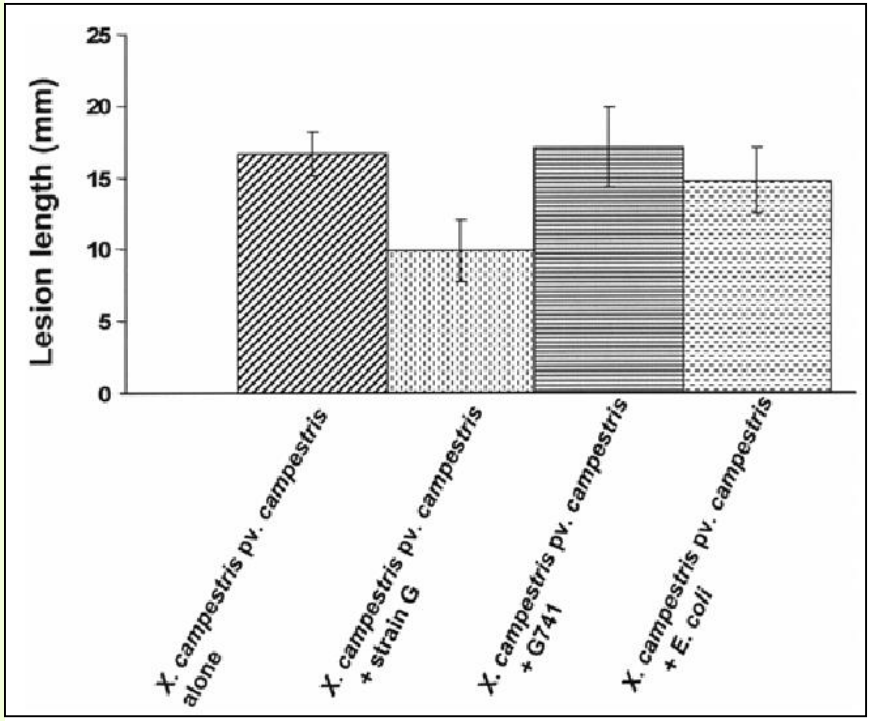
DSF disrupting/degrading bacteria

Xanthomonas campestris pv. *campestris*

- Severity of disease in mustard seedlings caused by *Xanthomonas campestris* pv. *campestris* when coinoculated with:

1. *Pseudomonas* spp. strain G
   or
2. With the *carAB* mutant G741.

- Lesion lengths were measured from the point of inoculation.



carAB genes are required for the synthesis of carbamoylphosphate, a precursor for pyrimidine and arginine biosynthesis is required for rapid degradation of DSF in strain G.

Newman *et al.*,2007

Mechanisms of QS inhibition

DSF disrupting/degrading bacteria

Xylella fastidiosa

- The *rpfF* homolog of *Xylella fastidiosa*, synthesizes a cell-to-cell signal similar to but apparently slightly different from that of *X. campestris* pv.*campestris*.
- Unlike *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae*, *Xylella fastidiosa* requires insect vectors for spread from plant to plant.
- Intriguingly, *Xylella fastidiosa rpfF* mutants exhibit increased virulence to plants but are unable to form a biofilm within insect vectors that acquire this mutant and, thus, cannot be spread from plant to plant by their insect vectors.
- Thus, as in *Xanthomonas* species, DSF perception also appears to play an important role in the biology of *Xylella fastidiosa*.

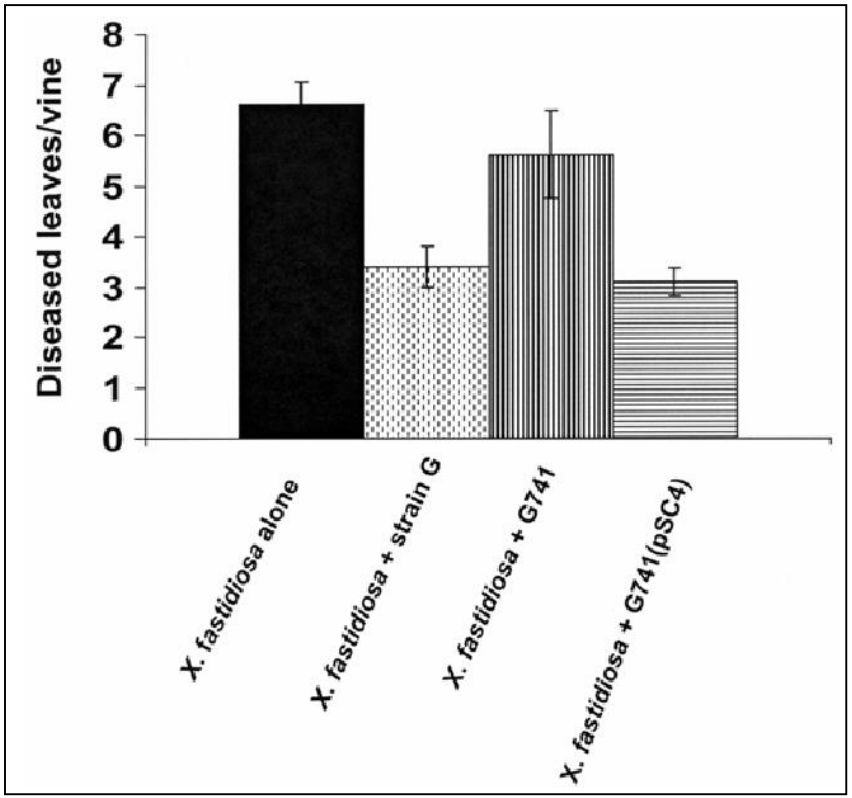
Newman *et al.*,2007

Mechanisms of QS inhibition

DSF disrupting/degrading bacteria

Xylella fastidiosa

- Severity of Pierce's disease of grape coinoculated with *Xylella fastidiosa* STL and diffusible signal factor–degrading:
  1. *Pseudomonas* spp. Strain G,
  2. *CarAB* mutant G741 of *Pseudomonas* spp. strain G, or
  3. With mutant G741 complemented with pSC4, as measured 3 months after inoculation.



Newman *et al.*,2007

Quorum-quenching (QQ)

B. Plant-based compounds

BB. Eukaryotes quorum sensing inhibitors

1. Algal compounds
2. Plant-made compounds
3. Fungal compounds
4. Animal compounds

Boyer and Wisniewski-Dyé,2009

Manipulation of QS

B. Plant-based compounds

Inter-kingdom signals

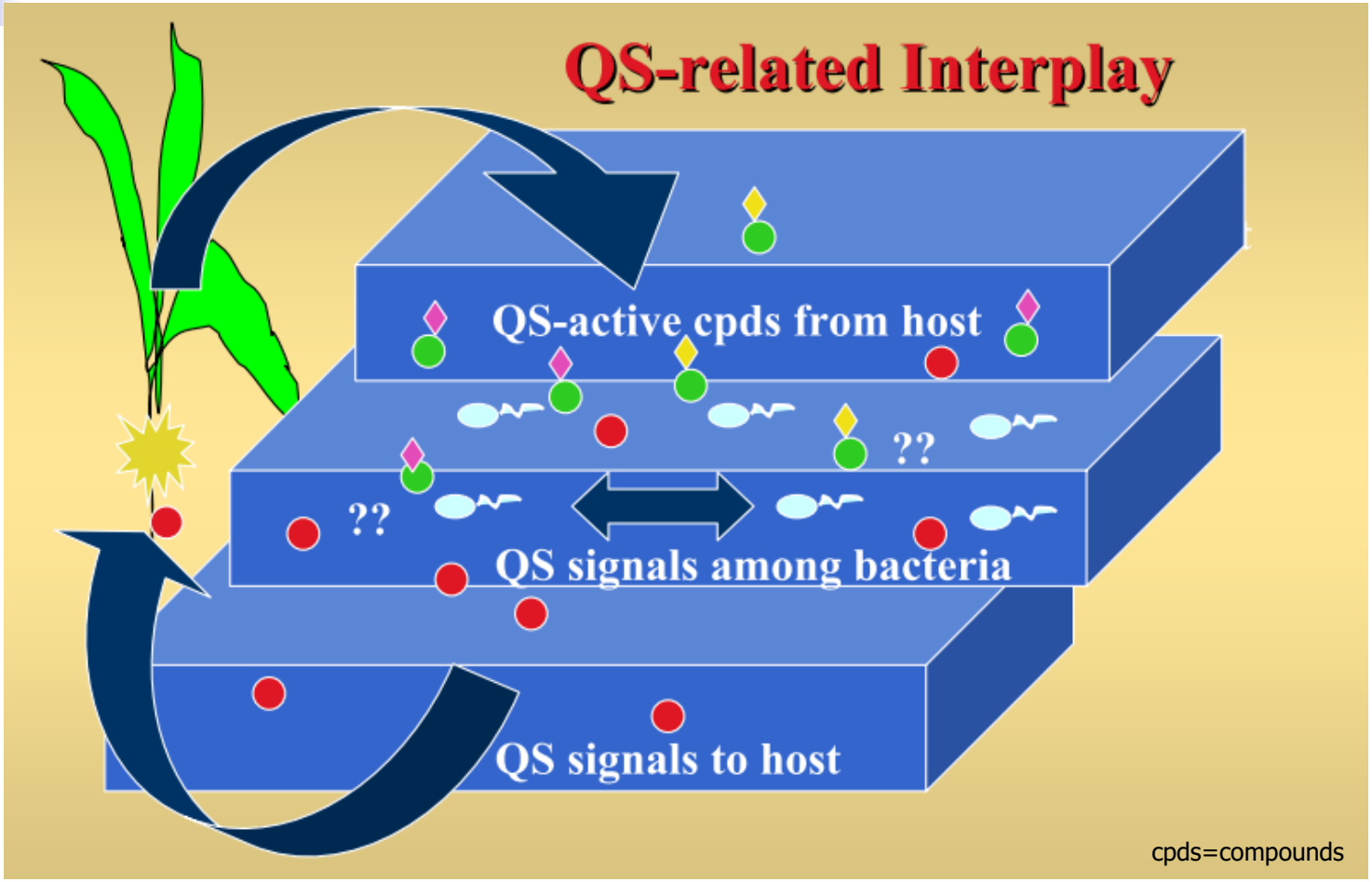
- Plants have evolved numerous chemical strategies for deterring pathogen attack, including the production of bactericidal and anti-infective compounds.
- Researchers from different countries trying to find new anti-QS compounds from their endemic plants.
- This helps some labs to establish a screening platform for endemic plants in order to find new anti-Qs molecules(phytochemicals).
- Unlike a conventional antimicrobial agent which attempts to control disease by its microbiostatic or microbiocidal effect on cells, an anti-QS compound works by causing an interruption of the QS mechanism of pathogens.

Adonizio *et al.*,2008; Tsiry Rasamiravaka;..

Manipulation of QS

B. Plant-based compounds

QS related Interplay



Robinson *et al.*,2003

Manipulation of QS

Plant-based compounds

Eukaryotic disruption of bacterial QS/biofilm

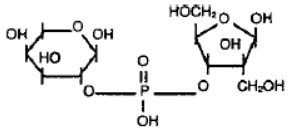
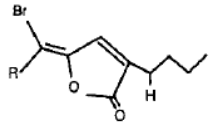
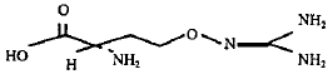
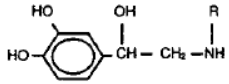
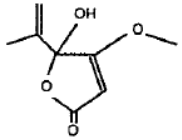
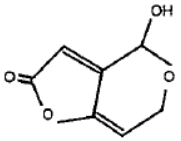
- **Plants as QS antagonists:**
- Many plant extracts such as fruit extracts of grape and strawberry, garlic, vanilla, lily and pepper acting as inhibitors of quorum sensing.
- The latex of several species of *Euphorbia* plants (spurges) is known to contain several di- and tri-terpenes and their esters which possess anti-infective properties (activities) but the mode of action is not yet clear.

Nashikkar *et al.*,2011; Faure *et al.*,2009..

Manipulation of QS

Plant-based compounds

Eukaryotic disruption of bacterial QS/biofilm

QSI compound(s)	Structure	R	Source	Quorum-sensing system affected
Agrocinopine B			Crown gall cells of host plants	Tra system of *A. tumefaciens*
Furanone		H or Br	*D. pulchra*	Swr system of *S. liquefaciens* and other gram-negative bacteria
L-Canavanine			*M. sativa*	Sin/ExpR system of *S. meliloti*
Norepinephrine, epinephrine		H or CH_3	Human hormones	AI-3 system of EHEC
Penicillic acid			*Penicillium* spp.	Las and Rhl systems of *P. aeruginosa*
Patulin			*Penicillium* spp.	Las and Rhl systems of *P. aeruginosa*

González and Keshavan,2006

Manipulation of QS

Phytochemicals with proved antiquorum sensing activity

Eukaryotic disruption of bacterial QS/biofilm

Molecules
Gamma aminobutyric acid (GABA)
Pyrogallol
Curcumin
Cynnamaldheyde
Furocoumarins
Flavanones, flavonoids, flavonols
Ursolic acid
Rosmarinic acid
Salycilic acid

Molecules
Epigallocatechin gallate, Ellagic acid, Tannic acid
Urolithin A and B
4,5-*O*-dicaffeoyl quinic acid
Chlorogenic acid, vanillic acid, proanthocyanidins
Volatile organic compounds
Furanones

Nazzaro *et al.*,2013

Manipulation of QS

Plant-based compounds

Antagonist of QS against selected bacteria and pathogens

- In recent years, the discovery of QS antagonists of bacterial and non-bacterial origin has increased tremendously.
- There are some chemically synthesized compounds that inhibit QS, but most of the antagonists have been discovered in plants extracts/essential oils.
- Table shows some of the antagonists discovered in recent studies.

Source	Antagonist	Inhibition against
Plant extracts	*Melicope lunu-ankenda* (leaves)	*E. coli* [pSB401]
	Syzygium aromaticum (bud)	*E. coli* [pSB1075]
		C. violaceum CV026
		P. aeruginosa PA01
		P. aeruginosa lecA::lux
	Garlic (bulbs)	*P. aeruginosa*
	Vanilla planifolia (beans)	*C. violaceum* CV026
	Tremella fuciformis (whole)	*C. violaceum* CV026
	Panax notoginseng (flowers and roots)	
	Areca catechu (seeds)	
	Prunus armeniaca (kernel of seed)	
	Prunella vulgaris (whole)	*C. violaceum* CV026
	Nelumbo nucifera (leaves)	*P. aeruginosa* PA01
	Punica granatum (bark)	
	Imperata cylindrical (stem)	
	P. ginseng (roots)	
	Moringa oleifera (leaves and fruits)	*C. violaceum* ATCC 12472
	Capparis spinosa (fruits)	*C. violaceum* CV026
		P. aeruginosa
		E. coli
		Proteus mirabilis
		Serratia marcescens
	Laurus nobilis (fruits, flowers, leaves, bark)	*C. violaceum* ATCC 12427
	Acacia nilotica (green pod)	*C. violaceum* ATCC 12472
	Quercus virginiana (leaves)	
	Chamaesyce hypericifolia (aerial)	*C. violaceum* ATCC 12472
	Tetrazygia bicolor (leaves)	*C. violaceum* CV026
	Conocarpus erectus (leaves)	*Agrobacterium tumefaciens*
	Bucida burceras (leaves)	NTL4
	Callistemon viminalis (leaves, inflorescence)	

Koh *et al.*,2013

Manipulation of QS

Plant-based compounds

Antagonist of QS against selected bacteria and pathogens

Source	Antagonist	Inhibition against
Plant extracts	*Vaccinium macrocarpon* *V. angustifolium* *Rubus idaeus* *R. eubatus* *Fragaria* sp. *Vitis* sp. *Origanum vulgare* *Rosemarinus officinalis* *Ocimum basilicum* *Thymus* sp. *Brassica oleracea* *Curcuma longa* *Zingiber officinale*	*C. violaceum* CV026 *C. violaceum* 31532 *P. aeruginosa* PA01 *E. coli* O157:H7
	Lonicera alpigena *Castanea sativa* *Juglans regia* *Ballota nigra* *R.officinalis* *Leopoldia comosa* *Malva sylvestris* *Cyclamen hederifolium* *Rosa canina* *R. ulmifolius*	*Staphylococcus aureus*
	Ananas comosus *Musa paradiciaca* *Manilkara zapota* *Ocimum sanctum*	*C. violaceum* ATCC 12472 *C. violaceum* CV026 *P. aeruginosa* PA01
	Scutellaria baicalensis	*C. violaceum* CV026
	Scorzonera sandrasica	*C. violaceum* ATCC 12472 *C. violaceum* CV026
	Orange	*Yersinia enterocolitica*
Essential oils	Tea tree Rosemary	*C. violaceum* CV026
	Lippia alba *Ocotea* sp. *Elettaria cardamomum* *Swinglea glutinosa* *Myntotachys mollis* *Zingiber officinale*	*P. putida* [pRK-C12) *E. coli* [pJBA132]
	Piper brademeyeri (leaves) *P. brachypodom* (leaves) *P. bogotence* (whole)	*C. violaceum* CV026
Bioactive metabolites	*Phellinus igniarius*	*C. violaceum* CV026

Source	Antagonist	Inhibition against
Plant exudates	Exudates from pea (*Pisum sativum*)	*Serratia liquefaciens* MG44 *S. faciens*MG44
Fungal extracts	*Ganoderma lucidum*	*C. violaceum* CV026
Broccoli	Sulforaphane Erucin	*P. aeruginosa*
Myristica cinnamomea *Combretum albiflorum*	Malabaricone C Catechin	*P. aeruginosa* PA01 *C. violaceum* CV026

Koh *et al.*,2013

Manipulation of QS

Quorum sensing inhibitors of eukaryotic origin

Algae and fungi

Source and quorum sensing inhibitor	Effective against	
	organism	Quorum sensing activity
Algae		
Ahnfeltiopsis flabelliformis (Korean red alga): α-D-galactopyranosyl-(1→2)-glycerol (Floridoside), betonicine and isoethionic acid	*Agrobacterium tumefaciens*	AHL mediated
Chlamydomonas reinhardtii, unicellular green alga: Unidentified AHL mimics	*Escherichia coli* (LasRI_::*luxCDABE*)	Bioluminescence
Delisea pulchra (Australian macroalga, Sea weed): Halogenated Furanone	*E. coli*	Biofilm formation and swarming AI-2 signaling system (chemotaxis, motility, and flagellar synthesis)
	Proetus mirabilis	Swarming motility
	Pseudomonas aeruginosa	Biofilm formation
	Serratia liquefaciens	Swarming motility
	Vibrio fischeri	Bioluminescence
	Vibrio harveyi	Toxin production and Luminescence Biofilm formation Bioluminescence
Laminaria digitata (Brown alga): Oxidized halogen HOBr	*Chromobacterium violaceum* CV026	Reacts specifically with 3-oxo-acyl HSLs
Fungi		
Tremella fuciformis (White jelly mushroom — basidiomycete): fruiting bodies	*C. violaceum*	Violacein production
Auricularia auricular: natural pigments	*C. violaceum*	Violacein production
Penicillium: patulin and penicillic acid	*P. aeruginosa*	Biofilm
Plants		
Allium sativum (garlic) extract	*A. tumefaciens* strain NTL4	β-galactosidase
	C. violaceum	Violacein production
	P. aeruginosa	Alginate and elastase Biofilm formation

Kalia,2013

Manipulation of QS

Quorum sensing inhibitors of eukaryotic origin

Plants

Plants		
Allium sativum (garlic) extract	*A. tumefaciens* strain NTL4	β-galactosidase
	C. violaceum	Violacein production
	P. aeruginosa	Alginate and elastase Biofilm formation
	P. aeruginosa strain IsoF/gfp	Fluorescence
Alyssum maritimum (leaf)	*C. violaceum* CV0blu	Slight inhibition
Ananas comosus	*C. violaceum*	Violacein production
	P. aeruginosa PAO1	Pyocyanin pigment, staphylolytic protease, ealstase production and biofilm formation
Arabidopsis exudate: γ-hydroxybutyrate (GHB)	*A. tumefaciens*	AHL signaling
Blueberry extracts	*C. violaceum*	Violacein production
Brassica napus (leaf)	*C. violaceum* CV0blu	Slight inhibition
Brassica oleracea, Basil, thyme, rosemary, ginger and turmeric: extracts of these herbs and spices	*C. violaceum*	Violacein production
Camellia sinensis (tea): catechins (Epigallocatechin gallate)	*E. coli*	Transfer of conjugative R plasmid
Cinnamomum zeylanicum (cinnamon), oil component-cinnamaldehyde	*P. aeruginosa* *E. coli*	Biofilm formation
	V. harveyi	AHL- and AI-2 mediated QS
Cinnamaldehyde and its derivative: 4-NO2-cinnamaldehyde	*Vibrio* spp.	AI-2-mediated QS — bioluminescence, protease activity, pigment formation
Combretam albiflorum: flavonoid catechin from bark	*P. aeruginosa*	C4-HSL perception by RhlR
Crown vetch	*C. violaceum* CV026	Violacein production
Grape extracts	*C. violaceum*	Violacein production
Grapefruit juice (furocoumarins)	*E. coli* *P. aeruginosa* *Salmonella typhimurium*	Biofilm formation
Lotus corniculatus (seedlings)	*A. tumefaciens* NTLR4	Beta-galactosidase
	C. violaceum CV026	Violacein production

Kalia,2013

Manipulation of QS

Quorum sensing inhibitors of eukaryotic origin

Plants

Lotus corniculatus (seedlings)	*A. tumefaciens* NTLR4	Beta-galactosidase
	C. violaceum CV026	Violacein production
Manilkara zapota	*C. violaceum*	Violacein production
	P. aeruginosa PAO1	Pyocyanin pigment, staphylolytic protease, ealstase production and biofilm formation
Musa paradiciaca	*C. violaceum*	Violacein production
	P. aeruginosa PAO1	Pyocyanin pigment, staphylolytic protease, ealstase production and biofilm formation
Medicago sativa seed exudate L-Canavanine, an arginine analog	*C. violaceum*	Violacein production
	Sinorhizobium meliloti	Exopolysaccharide II (EPSII)
Medicago truncatula (seedling fractions 8–12 and 21–24)	*E. coli*	LuxR reporter
		AhyR reporter
	C. violaceum	CviR reporter
M. truncatula	*P. aeruginosa*	QS in general
	Salmonella enterica	LuxR reporter to AI-2 signals
	S. meliloti 1021	QS in general

Kalia,2013

Manipulation of QS

Quorum sensing inhibitors of eukaryotic origin

Plants

Source and quorum sensing inhibitor	Effective against	
	organism	Quorum sensing activity
Ocimum basilicum (sweet basil): rosamarinic acid	*P. aeruginosa*	Protease and elastase production, biofilm formation and virulence factors
Ocimum sanctum	*C. violaceum*	Violacein production
	P. aeruginosa PAO1	Pyocyanin pigment, staphylolytic protease, ealstase production and biofilm formation
Passiflora incarnate (leaf)	*C. violaceum* CV0blu	Violacein production
Pisum sativum (seedlings)	*C. violaceum* CV026	C4HSL-inducible protese and N-acetylglucosaminidase (Exochitinase) Violacein production
	S. liquefaciens MG1	Swarming activity
P. sativum (roots)	*C. violaceum* CV0blu	Violacein production
P. sativum (leaf)	*C. violaceum* CV0blu	Slight inhibition
Raspberry extracts	*C. violaceum*	Violacein production
Romneya trichoclyx (leaf)	*C. violaceum* CV0blu	Slight inhibition
Ruta graveolens (leaf)	*C. violaceum* CV0blu	Slight inhibition
Scorzonera sandrasica extract	*C. violaceum* ATCC12472 and CV026	Violacein production
	Erwinia caratovora	Carbapenem antibiotic production
Squash exudate: γ-hydroxybutyrate (GHB)	*A. tumefaciens*	AHL signaling
Tomato seedlings exudate: γ-hydroxybutyrate (GHB)	*A. tumefaciens*	AHL signaling
Vanilla planifolia extract	*C. violaceum* CV026	Violacein production

Kalia,2013

Manipulation of QS

Quorum sensing inhibitors of eukaryotic origin

Medicinal plants

Medicinal plant extracts: *Conocarpus erectus* (leaves), *Chamaecyce hypericifolia* (aerial), *Callistemon viminalis* (leaves), *Bucida buceras* (leaves), *Tetrazygia bicolor* (leaves), *Quercus virginiana* (leaves)	*C. violaceum* *A. tumefaciens* NTL4 (pZLR4)	Violacein production Beta-galactosidase
Medicinal plant extracts: *C. erectus* (leaves), *C. viminalis* (leaves), *B. buceras* (leaves)	*P. aeruginosa*	Protease, elastase, pyoverdin production and biofilm formation
Traditional Chinese medicines	*A. tumefaciens* *P. aeruginosa*	TraR inhibitor Biofilm formation
Combretum albiflorum (bark)	*P. aeruginosa*	Biofilm formation and elastase
Secondary metabolites		
p-Coumaric acid (phenolic compound, a lignin precursor)	*C. violaceum* 5999 *Pseudomonas chlororaphis* *A. tumefaciens*	Violacein production Antibiotic phenazine-1-carboxylic acid (PCA) production Beta-galactosidase
Salicyclic acid (phenolic plant secondary metabolite)	*A. tumefaciens*	Stimulates AHL-lactonase expression
Vanillin	*Aeromonas hydrophila*	Biofilm formation
Manolide, manolide monoacetate and secomanoalide from marine sponge, *Luffariella variabilis*	*E. coli* (LuxR from *V. fischeri*) *P. aeruginosa*	Beta-galactosidase and Ttrazolium red
Animal		
Mammalian paraoxonases	*P. aeruginosa*	Reduction in 3OC12HSL dependent activity

Kalia,2013

Manipulation of QS

Plant-based compounds

Eukaryotic disruption of bacterial QS/biofilm

1. **Marine algae(QS signal-mimics):**

- Bacterial disease is one of the most critical problems in commercial aquaculture.
- A novel and environmental-friendly approach in solving this problem is through the disruption of bacterial communication or quorum sensing (QS).
- Several findings have reported that numerous aquatic organisms such as micro-algae, macro-algae, invertebrates, or even other bacteria have the potential to disrupt QS.
- Unicellular green algae make a number of inhibitory QS signal-mimics.
- Anti-QS compounds are known to exist in marine algae and have the ability to attenuate bacterial pathogenicity.

Natra *et al.*,2011; Adonizio *et al.*,2006

Manipulation of QS

Plant-based compounds

Eukaryotic disruption of bacterial QS/biofilm

- *Fucus* is a genus of brown algae found in the rocky seashores almost throughout the world.
- One of the common brown seaweed alga is *Fucus vesiculosus*.
- We tested crude extracts of 59 species of macroalgae from the Baltic and the North Sea.
- Therein extracts of 34 species showed activity in the anti settlement assay (biofilm development).
- These results indicate that algae can control the colonization process by release of repellents.

Wahl,2011

Manipulation of QS

Plant-based compounds

Eukaryotic disruption of bacterial QS/biofilm

- The discovery that the red marine alga (*Delisea pulchera*) produces furanone inhibitors of bacterial QS stimulated a search for similar activities in plants.
- The macro-algae produces compounds known as furanones, which can specifically interfere with AHL-mediated QS systems.
- **Furanones (QS signal-mimics) affect the:**

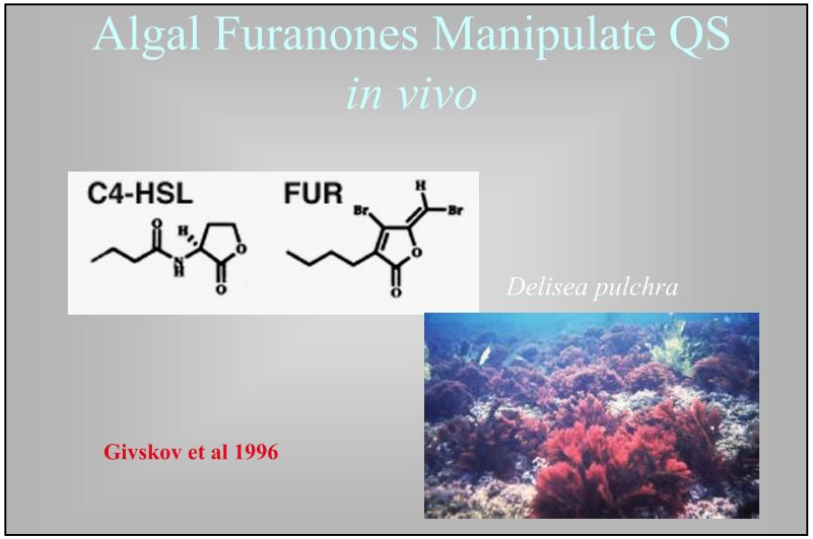
1. Growth of Gram-positive bacteria,
2. Inhibit quorum sensing, and
3. Swarming motility of marine bacteria.

- The use of natural products such as furanones, which target specific bacterial signaling and regulatory systems, represents a promising approach to inhibition of biofilm.

Nandiraju,2004;..

Manipulation of QS
Plant-based compounds
Similar chemical structures of furanones with AHLs

- Many furanones with chemical structures similar to the *N*-acylhomoserine lactones are produced in nature.
- Therefore many natural and synthetic furanones acting as agonists or antagonists were used as anti-QS compounds.



Robinson *et al.*,2003;Martinelli *et al.*,2004

Manipulation of QS

Plants produce substances that mimic AHLs

Chemical similarities between AHLs and furanone

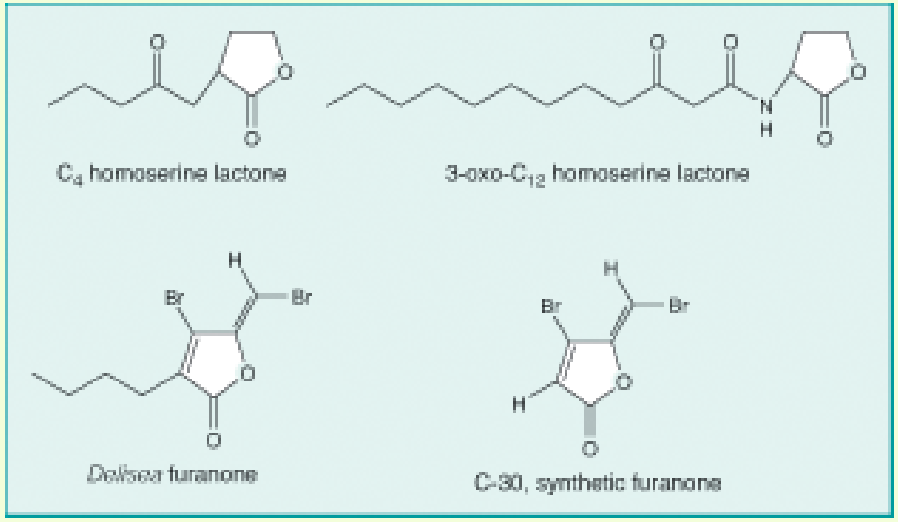
- Chemical similarities between AHLs and furanone mimics.

A. C4-homoserine lactone.

B. 3-oxo-C12-homoserine lactone.

C. One of the 30 natural halogenated furanones from *Delisea pulchra*.

D. A synthetic analog of the *Delisea* furanones.

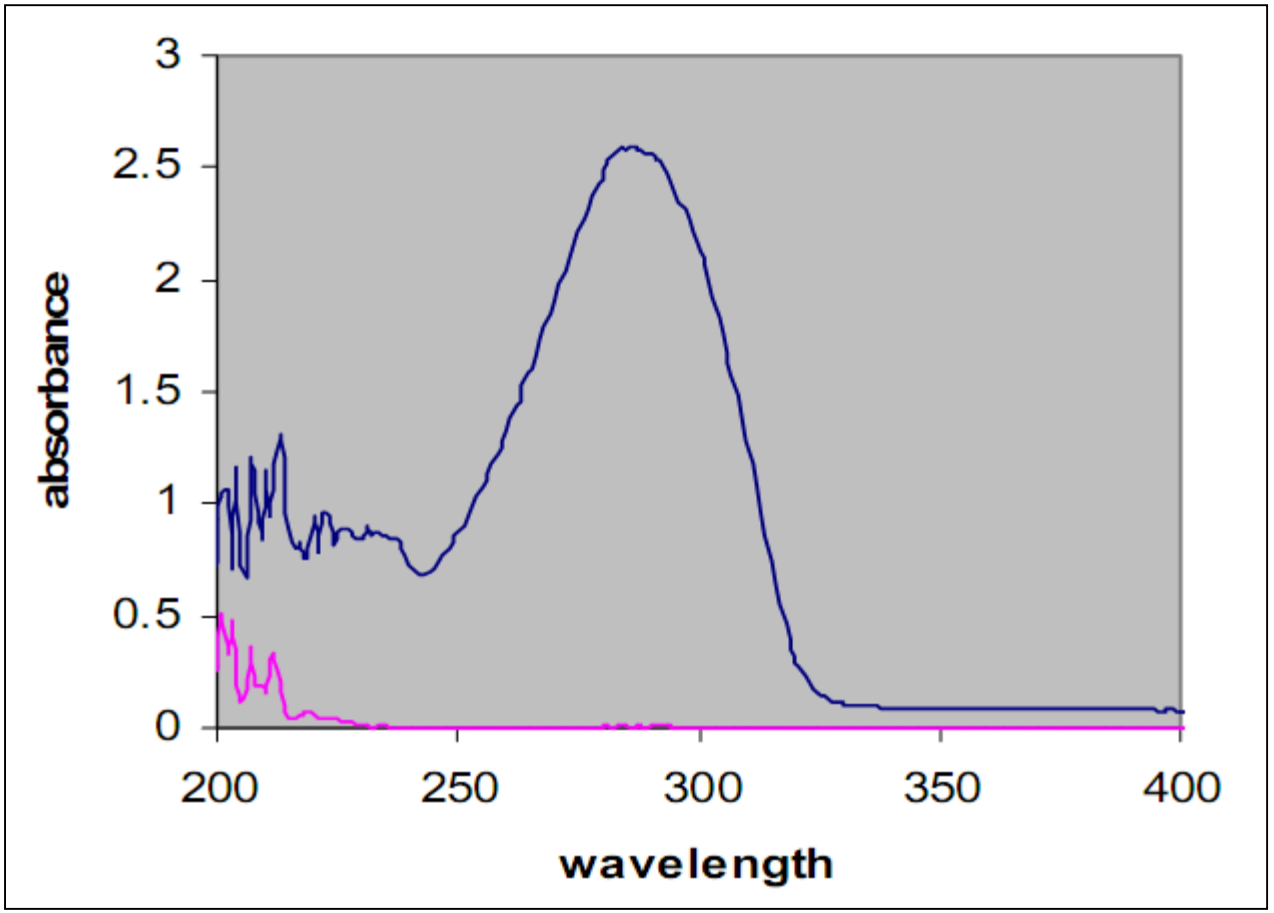


Bauer *et al.*,2005

Manipulation of QS

Plant-based compounds

UV spectrum of the furanones fimbrolide (21)



Nandiraju,2004

Manipulation of QS

Plant-based compounds

Signals antagonized by furanones

- **Marine Australian macroalgae (*Delisea pulchera*):**
- Cross talk between different signalling systems:

Organism	Signal type	Cross talk in AHL systems	Signals antagonized by furanones
Pseudomonas spp.	1. AHL, 2. cyclic dipeptide (CDP)	yes yes	yes Yes
Escherichia coli	cyclic dipeptide (CDP)	yes	Yes
Serratia liquefaciens	AHL	yes	yes
Vibrio angustum S14	unknown	yes	yes
Vibrio vulnificus	unknown	Yes	yes

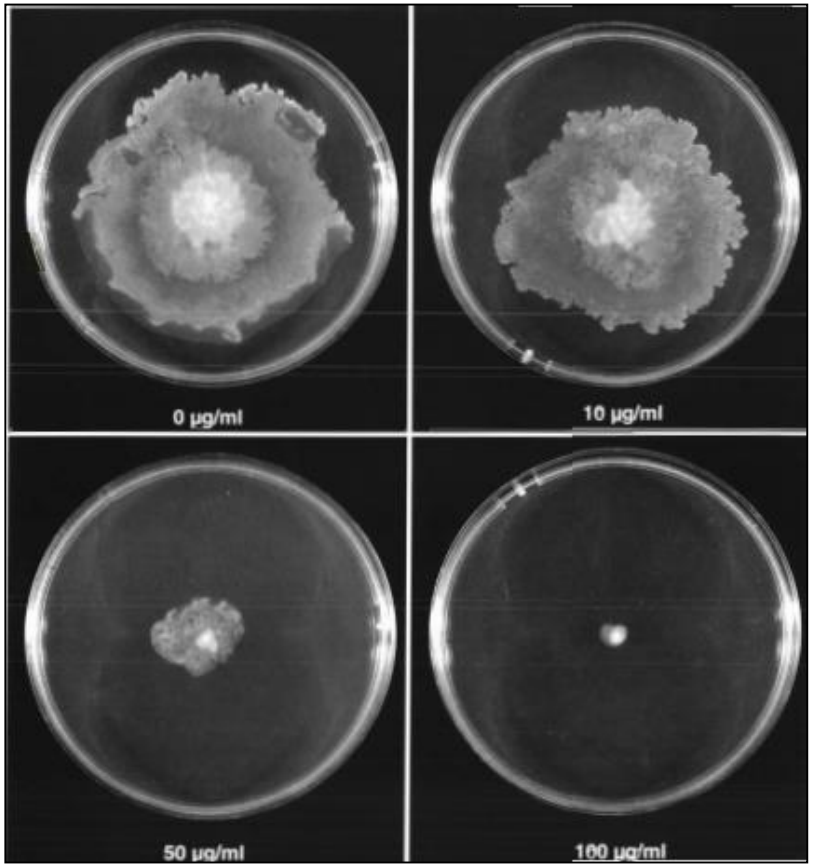
de Nys et al

Manipulation of QS

Plant-based compounds

Effect of furanone 2 on swarming motility of *Serratia liquefaciens*

- Effect of increasing concentrations (0, 10, 50, 100 µg ml^{-l}) of *Delisea pulchra* furanone 2 on *Serratia liquefaciens* swarming motility.
- Agar plates were stab inoculated at the center from an exponentially growing culture(OD_{450} of approximately 0.5) and incubated at 30°C.
- Increasing concentrations of furanone 2 progressively reduces the speed by which the swarming colony expands.



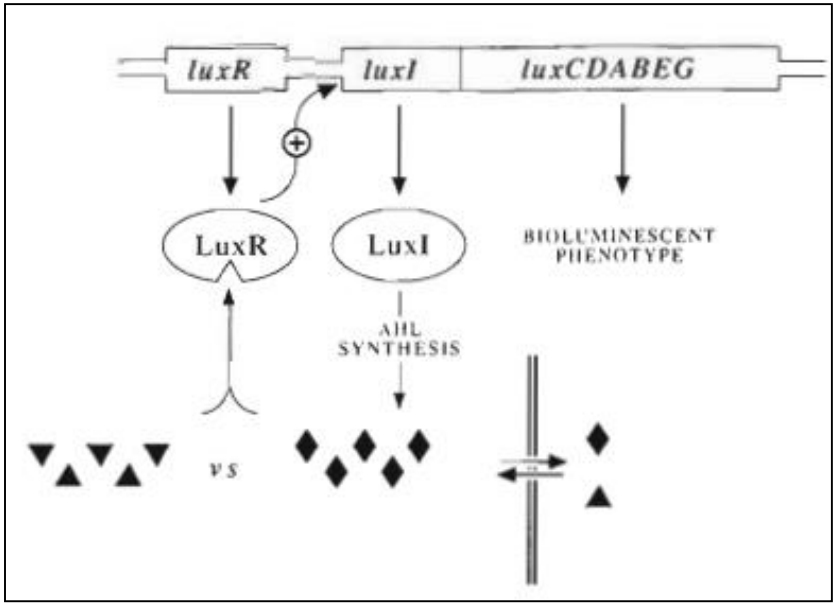
Givskov *et al.*,1996; Kjelleberglv *et al.*,1997

Manipulation of QS

Plant-based compounds

Effect of furanone 2 on swarming motility of *Serratia liquefaciens*

- Diagrammatic description of the inhibition of AHL mediated gene transcription by furanones of the alga *Delisea pulchera* as illustrated through the model bioluminescent *Lux* system of Vibrio *fischeri*.
- Furanones (▲) are proposed to compete with AHLs (◆) for a binding site on the LuxR protein interfering with transcription of the *1uxICDABEG* operon.



Kjelleberglv *et al.*,1997

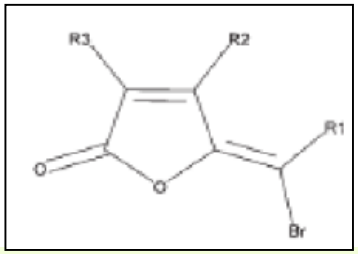
Manipulation of QS

Plant-based compounds

Effect of furanone on *P. carotovorum*

- The QSI molecule furanone (4-bromo-5-(bromomethylene)-3-(1‘-hydroxybutyl)-2-(5H)-furanone) has been shown to inhibit:

1. Antibiotic production, and
2. Many extracellular degradative enzymes (pectate lyases, cellulases and proteases) in the plant pathogen *Erwinia carotovora*.

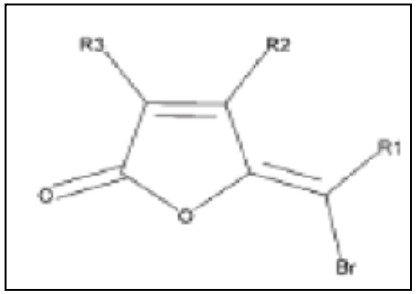


Halogenated furanone

Nagy,2010

Manipulation of QS
Plant-based compounds
Effect of furanone on *P. aeruginosa*

- Confocal microscopy demonstrated that this furanone helped reduced biofilm thickness (after 7 days) from 61±6 μm (untreated) to 23±4 μm (treated), and it lowered QS-mediated gene expression, as measured using fluorescent markers.



Halogenated furanone

Nagy,2010

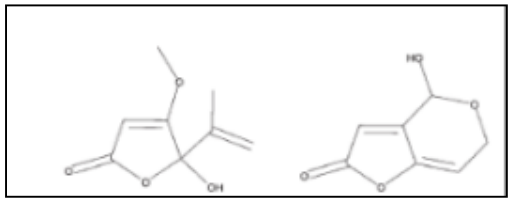
Manipulation of QS

Plant-based compounds

Eukaryotic disruption of bacterial QS/biofilm

2. Fungi:

- QSI compounds also were produced by Fungi.
- Rasmussen *et al.*,2005a studied 100 extracts from 50 *Penicillium* species and found that 33 produced QSI compounds.
- From these 100 extracts, two QSI molecules penicillic acid (Fig. left) and patulin (Fig. right) proved to be inhibitory against *P. aeruginosa* QS-controlled gene expression.



Nagy,2010

Manipulation of QS

Plant-based compounds

Eukaryotic disruption of bacterial QS/biofilm

3. Crop plants (pea, crown vetch and tomato):

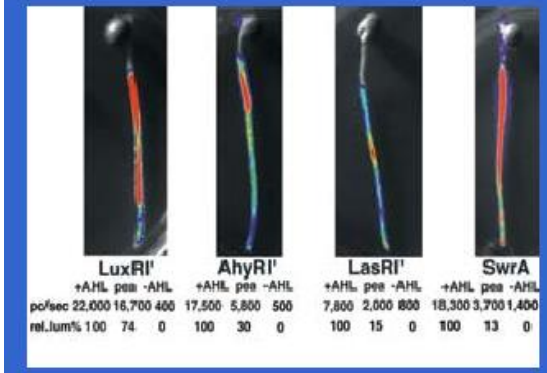
- Plant extracts have also been examined for QSI activity.
- Teplitski *et al.*,2000 have shown that higher plants such as pea, crown vetch and tomato all produce unidentified compounds that are capable of interacting with AHL-dependent QS systems.
- More recently, various species of plants including pea seedlings, garlic, vanilla, and L-canavanine, which is made by *Medigo sativa* have also been found to be able to interfere with bacterial QS.

Manipulation of QS

Plant-based compounds

Crop plants (pea, rice and tomato, soybean, alfalafa)

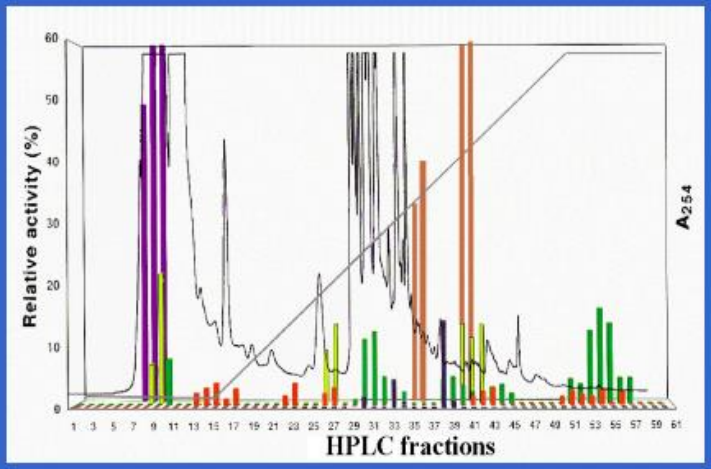
Plants secrete QS-active compounds that target bacterial AHL receptors



(Pea, rice, tomato, *M. truncatula*, soybean)

Teplitski et al 2000

Pea root exudate



Robinson *et al.*,2003

Manipulation of QS

Plant-based compounds

Crop plants (alfalfa)

- AHL signal mimics from *Medicago truncatula*.
- *M. truncatula* produces at least 12 dozen separable QS active compounds, most of them agonists.
- Different QS active compounds are secreted at different times during seedling development.
- The secreted QS active compounds often differ from those present inside the plant tissues (Gao *et al.*,2003).

Agonist: A substance that acts like another substance and therefore stimulates an action. Agonist is the opposite of antagonist.

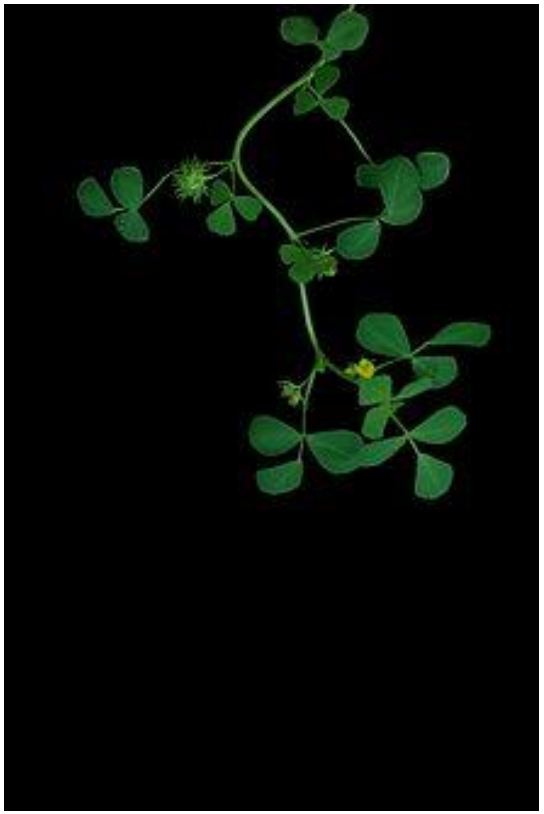
Robinson *et al.*,2003

Manipulation of QS

Plant-based compounds

Crop plants (alfalfa)

- *Medicago* is a genus of flowering plants, commonly known as medick or burclover.
- *Medicago sativa* is the best known member of the genus is alfalfa (also called lucerne).
- The species *Medicago truncatula* (Barrel Medic or Barrel Medick or Barrel Clover) is a model legume due to its relatively small stature, genome (450–500Mb), that is used in genomic research.
- It forms symbioses with nitrogen-fixing rhizobia and arbuscular mycorrhizal fungi.



Medicago truncatula

Wikipedia,2012

Manipulation of QS

Plant-based compounds

Crop plants (alfalfa)

- At least 10 to 20 chromatographically separable active compounds can be detected in root exudates of both pea and *Medicago truncatula*, a close relative of alfalfa.
- These plant compounds affect certain AHL receptors but not others.
- Thus, these plants produce a diversity of compounds that appear to be "AHL signal mimics" i.e., compounds that specifically affect AHL-mediated QS in bacteria but are chemically different from bacterial AHLs.
- AHL mimic compounds capable of disrupting QS in the bacteria they encounter.

Adonizio *et al.*,2008

Manipulation of QS

Plant-based compounds

Proteome analysis

- Proteome analysis of responses to AHL QS signals in *Medicago truncatula* (Mathesius *et al.*,2003):
- Exposed roots to AHLs at 1 nM - 100 nM initial concentration:
- 3-oxo-C12-HSL (*Pseudomonas aeruginosa*)
- 3-oxo-C16:1-HSL (*Sinorhizobium*)
- Extracted root proteins after 24 h or 48 h, 2D gel separation, MS protein identification.
- Of ~ 2,000 root proteins resolved on gels, ~ 7% showed differential accumulation in response to the AHLs.

Robinson *et al.*,2003

Manipulation of QS

Inhibition of quorum sensing responses

Crop plants (alfalfa)

A. Compounds secreted by alfalafa(*Medicago truncatula*) complement an AHL synthase mutation in *Serratia liquifaciens* and stimulate QS-dependent surface swarming.
B. The same *Serratia* synthase mutant was not stimulated to swarm when incubated under the same conditions on a moist toothpick.
C. AHL mimics from the plant activate a QS-regulated lux fusion in associated *Serratia* cells.

The wild *Serratia iquefaciens* MG1 displays swarming motility when exposed to a semi-solid surface. Swarming motility is under control of QS and regulated by *swrA* gene and its product, the surface-active compound serrawettin W2.

Manipulation of QS

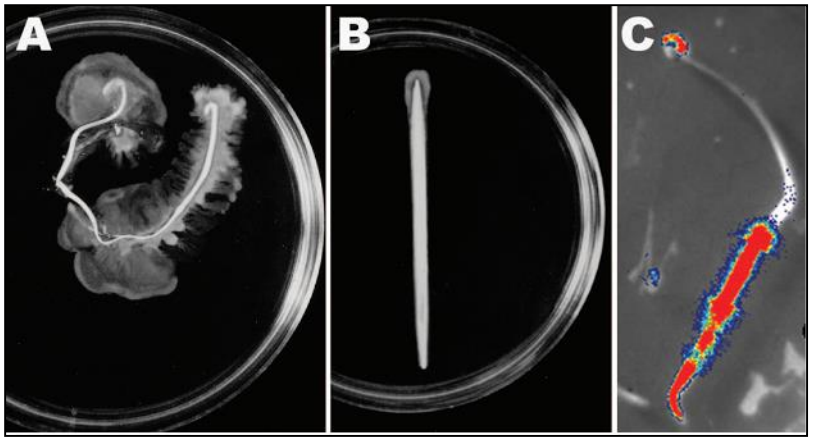
Inhibition of quorum sensing responses

Crop plants (alfalfa)

A. Compounds secreted by *Medicago truncatula* complement an AHL synthase mutation in *Serratia liquifaciens* and stimulate QS-dependent surface swarming.

B. The same Serratia synthase mutant was not stimulated to swarm when incubated under the same conditions on a moist toothpick.

C. AHL mimics from the plant activate a QS-regulated lux fusion in associated *Serratia* cells (false color image: red yellow green blue).

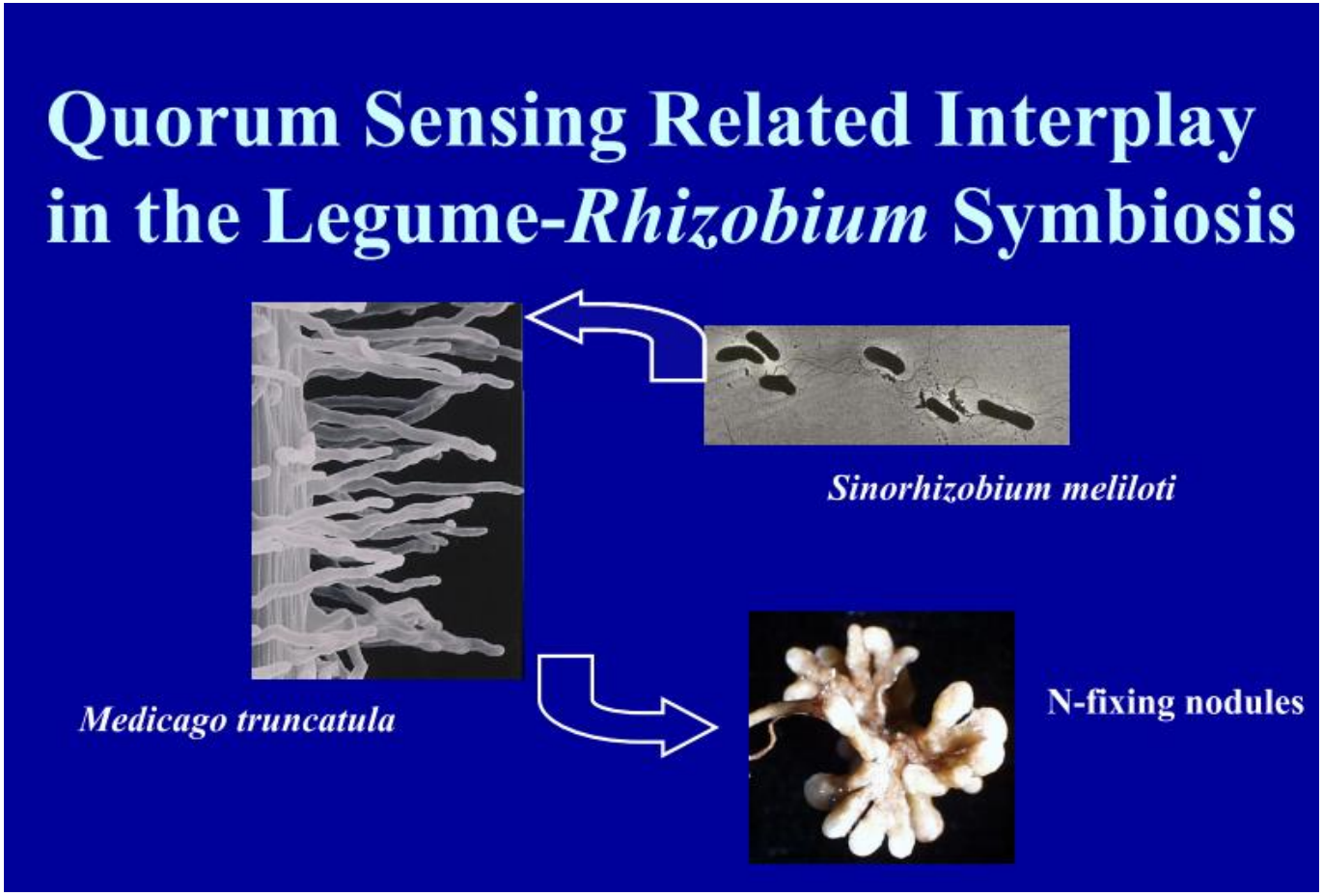


Bauer *et al.*,2005

Manipulation of QS

Plant-based compounds

Crop plants (alfalfa)



Robinson *et al.*,2003

Manipulation of QS

Plant-based compounds

Quorum sensing related interplay in the legume-*Rhizobium* symbiosis

- Characteristics of *Sinorhizobium meliloti*:
- Heterotrophic soil bacterium.
- Very versatile metabolism-can use a wide variety of compounds for carbon and energy.
- Fairly robust (vigor).
- Good genetic tools for this organism.

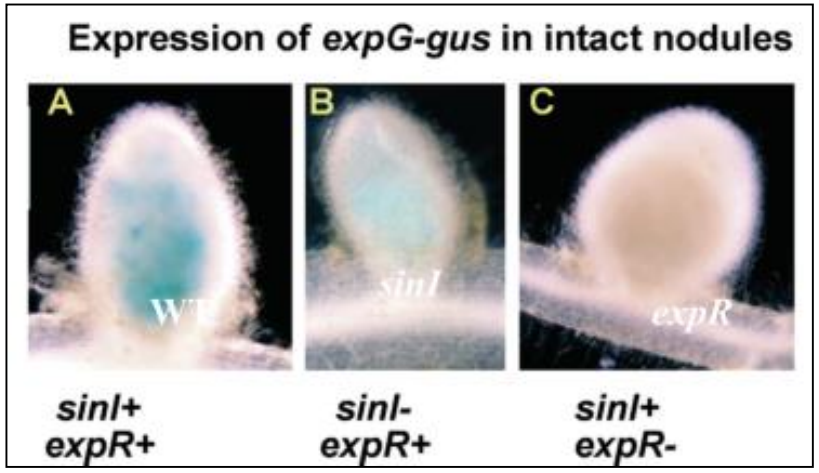
Robinson *et al.*,2003

Manipulation of QS

Plant-based compounds

QS agonists (mimics) from *M. truncatula* may affect *S. meliloti* QS *in planta*

- The work of Keshavan and colleagues in 2005 found that L-canavanine, secreted by the legume alfalfa (*Medicago sativa*) interferes with the QS of *Sinorhizobium meliloti*, a nitrogen-fixing bacterium that invade its roots.
- *expG* regulates EPSII synthesis. EPSII is a symbiotic signal.
- *expG* is QS-regulated by SinI AHLs via the ExpR receptor.



beta-glucuronidase (gus) gene. AHL synthase sinI and an AHL-regulated gene, expG, were activated inside the nodules.

Wikipedia,2012;Robinson *et al.*,2003

Manipulation of QS

Eukaryotic disruption of bacterial QS/biofilm

Crop plants (garlic)

- Persson *et al*.,2005 reported that toluene extracts of medicinal folklore, *Allium sativum* L., commonly known as garlic contained several compounds with varying levels of quorum sensing inhibition against Gram-negative transcriptional regulators Lux R or Las R of *P. aeuroginosa*.
- Specifically, N-heptysulfanylacetyl–L-homoserine lactone, a synthetic derivative, showed QSI activity against both Lux I/R and Las I/R QS mediated systems of *P.aeuroginosa*.
- Two compounds that were isolated from garlic inhibited QS in a LuxR reporter assay.

AL-Ghonaiem *et al*.,2009

Manipulation of QS

Eukaryotic disruption of bacterial QS/biofilm

Crop plants (garlic)

- The impact of QS in the regulation of swimming motility of *Agrobacterium tumefaciens*:
- We found that treatment of *A. tumefaciens* culture with different exogenous QS compounds(AHLs) induced swimming motility.
- Garlic bulb and *Salvadora persica* extracts were investigated for their QQ activity.
- While *S. persica* extract did not show any significant QQ activity, garlic bulb extract showed QQ activity against C4 AHL, C8 AHL, C10 AHL and C14 AHL, repressing the *A. tumefaciens* swimming motility induced by these QS compounds.

AL-Ghonaiem *et al.*,2009

Manipulation of QS

Swimming motility in *A. tumefaciens* is controlled by quorum sensing and inhibited by garlic bulb extract

Swimming motility assay of *A. tumefaciens*

- 100 µl of AHL solutions were added to 30 mL sterile glass tubes and the solvent e.g. acetic acid-acidified ethyl acetate was evaporated in a 35°C water bath before the addition of 10 mL of LB broth to give a final concentration of 10 µM for the QS compounds.
- *Agrobacterium tumefaciens* was grown in LB broth and incubated at 28°C with shaking at 250 rpm for 24 h.
- This culture was used to inoculate the QS compound-containing media and the resulting culture was incubated at 28°C with shaking at 250 rpm for 24 h.
- These cultures were then used for the swimming motility assays.

AL-Ghonaiem *et al.*,2009

Manipulation of QS

Swimming motility in *A. tumefaciens* is controlled by quorum sensing and inhibited by garlic bulb extract

Swimming motility assay of *A. tumefaciens*

- The swimming migration assay was performed as described previously (Atkinson *et al.*,2006).
- Briefly, 5 µl of *Agrobacterium tumefaciens* overnight cultures (grown in LB media or LB media containing different QS compounds), were inoculated onto the center of a 0.4% soft LB agar plate and then incubated at 28°C.
- The swimming migration distance was assayed by following the colony fronts of the bacterial cells.
- Progress was recorded at 60 min intervals for 48 h.

AL-Ghonaiem *et al.*,2009

Manipulation of QS

Swimming motility in *A. tumefaciens* is controlled by quorum sensing and inhibited by garlic bulb extract

Swimming motility assay of *Agrobacterium tumefaciens*

- Garlic bulb and *Salvadora persica* extract preparation:
- Briefly, garlic bulbs and *Salvadora persica* were cut into small pieces and dried in a plant drier for approximately 24 h at room temperature.
- Dried plant materials was ground and added to 95% ethanol (100 g dry wt. L^{-1}) and allowed to stand for 24 h before vacuum filtration with Whatman filter paper (No. 1) to remove particulate materials.
- The solution was evaporated to dryness using a rotary evaporator.
- The dry materials were stored at -20°C and reconstituted as needed in 95% ethanol and filtered through a 0.22 µm-pore-size filter membrane (Millipore).

AL-Ghonaiem *et al.*,2009

Manipulation of QS

Swimming motility in *A. tumefaciens* is controlled by quorum sensing and inhibited by garlic bulb extract

Inhibition of *A. tumefaciens* swimming by garlic extract

- **Anti-Quorum Sensing Activity:**
  For anti-QS activity testing, garlic bulb or *S. persica* extract was mixed with different QS compounds(AHLs) in LB broth (5 mL) at a final concentration of 1 mg mL^{-1} and 10 µM, respectively and incubated for 10 min at room temperature with periodic shaking.
- LB media with only QS compounds, LB media with only garlic extract and LB media with only *S. persica* were used as controls.
- All media were inoculated with overnight cultures of *Agrobacterium tumefaciens* and incubated at 28°C with shaking at 250 rpm for 24 h.

AL-Ghonaiem *et al.*,2009

Manipulation of QS

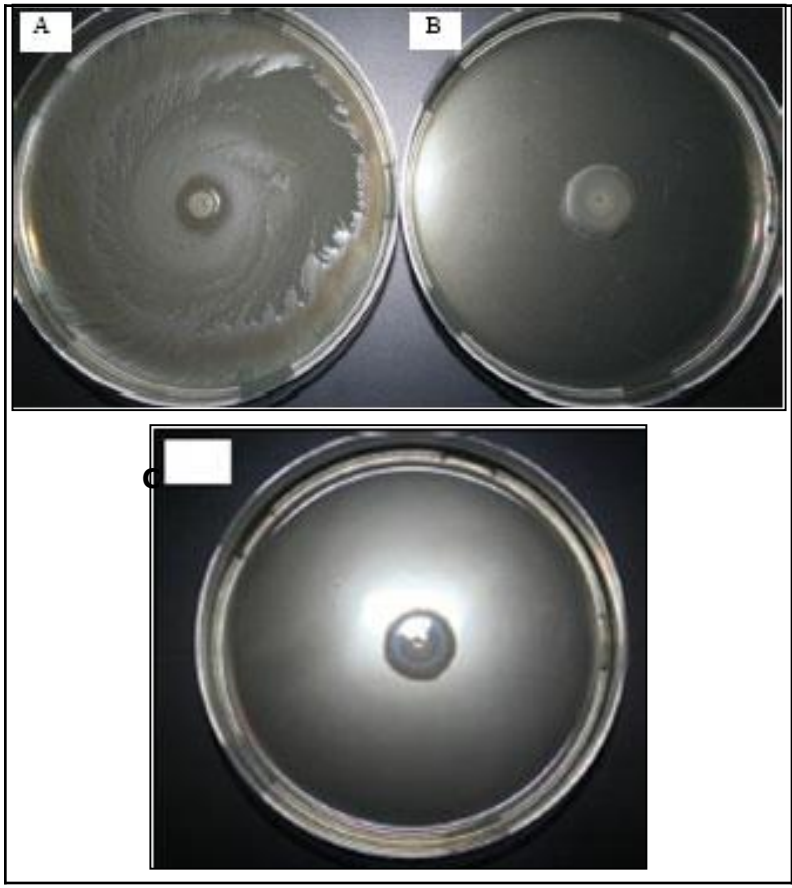
Swimming motility in *A. tumefaciens* is controlled by quorum sensing and inhibited by garlic bulb extract

Inhibition of *A. tumefaciens* swimming by garlic extract

A. Cells treated with C8 AHL. Induction of bacterial swimming motility after only 24 h of incubation

B. Cells treated with mixture of C8 AHL and garlic bulb extract. Inhibition of *Agrobacterium tumefaciens* vortex swimming by garlic extract.

C. Control (with no QS compound treatment). Limited cell swimming only after 4-5 days of incubation.



AL-Ghonaiem *et al.*,2009

Manipulation of QS

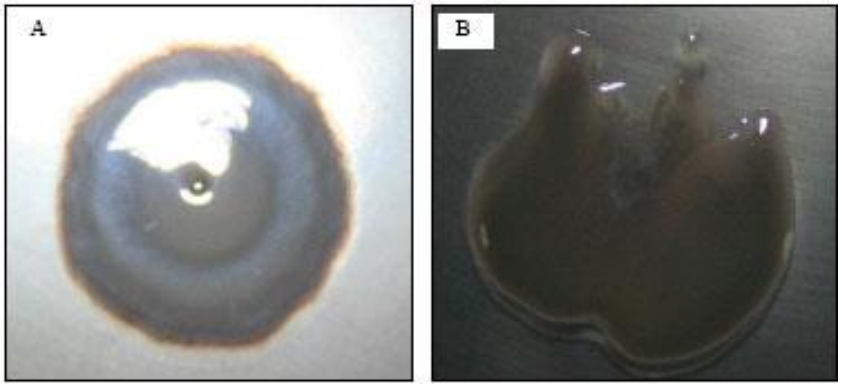
Excess slime production

C6 AHL

- C6 AHL, induced excess slime production and brown pigmentation of the *A. tumefaciens* colony.

A. Control,

B. *A. tumefaciens* treated with C6AH.



Induction of polysaccharide production by C6AHL.

AL-Ghonaiem *et al.*,2009

Manipulation of QS

Anti-QS activity in medicinal plant

Eukaryotic disruption of bacterial QS/biofilm

4. Medicinal plants:

- According to the WHO, 70-80% of the world's population still relies on folk-medicinal medicine(traditional medicines) as part of their main form of medical treatments (WHO,2008).
- Twenty-five percent of all drugs on the market have at least one compound derived from a plant source.
- If fungal and animal sources are included, the number jumps to 40% (Houghton,2001).

Nagy,2010

Manipulation of QS

Anti-QS activity in medicinal plant

Eukaryotic disruption of bacterial QS/biofilm

- **Medicinal plants:**
- Terrestrial plants traditionally used as medicines may also produce anti-QS compounds(QSIs).

1. Quorum sensing inhibitors(QSIs) reduce microbial virulence by interrupting quorum communication.
2. Also reduce or prevent the development of biofilm formation.
3. Several compounds have been identified that have the ability to interfere with QS-mediated gene expression through competitive inhibition, thus reducing biofilm thickness.

Nagy,2010

Manipulation of QS

Anti-QS activity in medicinal plant

Eukaryotic disruption of bacterial QS/biofilm

- Cell-to cell communication system in *P. carotovorum* has signal molecules at 3-oxo-C6-HSL, whereas in *R. solanacearum* at C8-HSL(Cragg *et al.*,1997).
- Anti-QS activity of some medicinal plants against:

1. *P. carotovorum*
2. *R. solanacearum.*

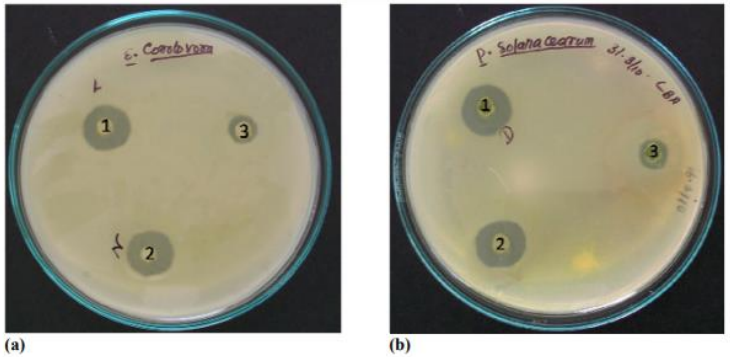


Figure 1: -QS activity of (a) *E. carotovora* using Ethanolic Plant extracts *(1) Solanum indicum (madurai), (2) Solanum xanthocarpum and (3)* Control (ethanol). *(b) P. solanacearum* Ethanolic Plant extracts (1) *Moringa oleifera, (2) Phyllanthus niruri* and *(3)* Control (ethanol).

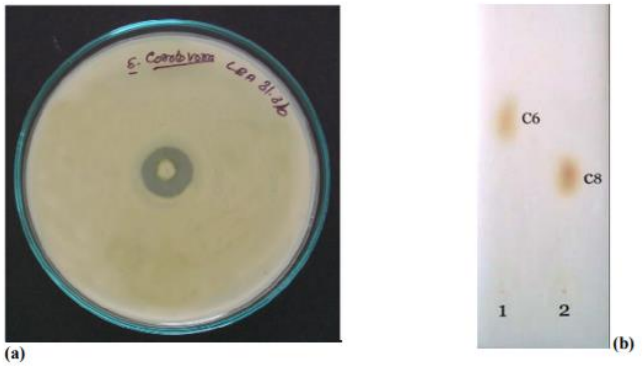


Figure 2: a) Carbapenem activity of *E. carotovora* in *E. coli* plate. (b) Separation and detection of AHL compound by thin layer chromatography showing the position of signal molecules at C6 and C8 in (1) *E. carotovora* and (2) *P. solanacearum* respectively.

Kumar *et al.*,2010

Manipulation of QS

Anti-QS activity in medicinal plant

Eukaryotic disruption of bacterial QS/biofilm

- **Medicinal plants:**
- 50 medicinal plants from southern Florida were screened for anti-QS activity using two biomonitor strains, *Chromobacterium violaceum* and *Agrobacterium tumefaciens*.
- Of these plants, six showed QS inhibition:
- *Conocarpus erectus*
- *Chamaecyce hypericifolia*
- *Callistemon viminalis*
- *Bucida burceras*
- *Tetrazygia bicolor*, and
- *Quercus virginiana.*

Adonizio *et al.*,2006

Methods

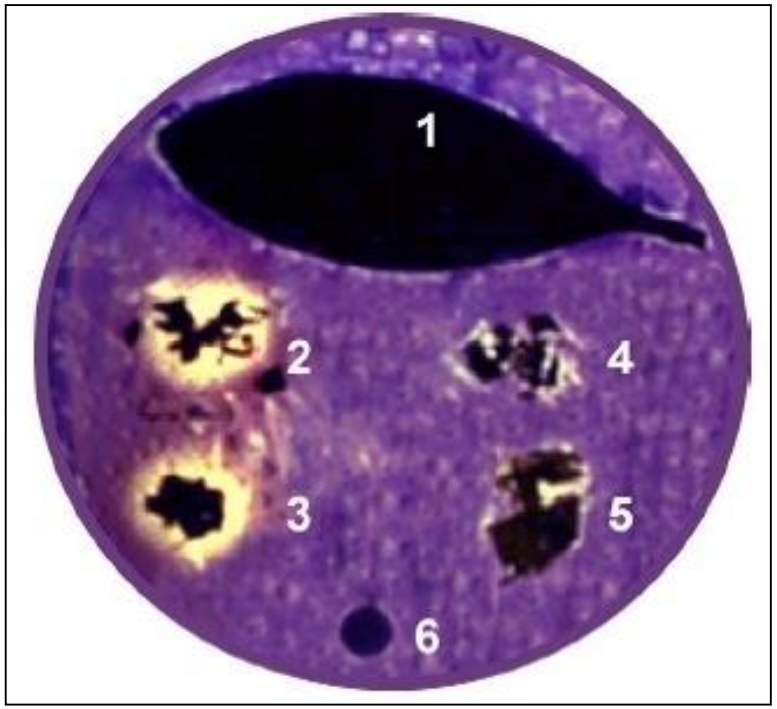
Preliminary screening of *Conocarpus erectus* for anti-QS activity using *Chromobacterium violaceum*

- Preliminary screening of *Conocarpus erectus* for anti-QS activity using *Chromobacterium violaceum* biomonitor strain.
- A yellowish "halo" of bacteria indicates an anti-QS effect.
- The samples were:

1. Washed whole leaf,
2. Washed macerated leaves, and
3. Unwashed macerated leaves of fresh *Conocarpus erectus*.

- Negative controls:

4. Washed,
5. Unwashed leaves of *Hamelia patens*, and
6. Sterile paper disk were included.



Adonizio *et al.*,2006 and 2008

Methods

Preparation of plant extracts with organic solvents such as hexane, chloroform, ethyl acetate, and ethyl alcohol

Choice of Solvents

- Successful determination of biologically active com-pound from plant material is largely dependent on the type of solvent used in the extraction procedure.
- Proper-ties of a good solvent in plant extractions include low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or dissociate.
- As the end product in extraction will contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassay.

Gurjar *et al.*,2012

Methods

Preparation of plant extracts with organic solvents such as hexane, chloroform, ethyl acetate, and ethyl alcohol

Choice of Solvents

- Solvents used for active component extraction.

Water	Ethanol	Methanol	Chloroform	Dichloro-methanol	Ether	Acetone
Tannins	Alkaloids	Terpenoids	Terpenoids	Terpenoids	Alkaloids	Flavonols
Saponins	Tannins	Saponins	Flavonoids	-	Terpenoids	-
Terpinoides	Terpinoides	Tannins	-	-	Coumarins	-
-	Flavonol	Flavones	-	-	-	-

Gurjar *et al.*,2012

Preparation of AHL stock solutions
The solvents

- Stock solutions (1 mM) of QS compounds such as C4 AHL, C6 AHL, 3oxo-C6 AHL, etc. were prepared in acetic acid-acidified ethyl acetate (0.01% vol/vol) (BDH) (HPLC grade) except C14 AHL, which was prepared in dichloromethane (BDH) solvent (HPLC grade).
- The stock QS solution was sterilized by filtration using a 0.22 µm-pore-size filter membrane (Millipore).

AL-Ghonaiem *et al.*,2009

AHL has to be dissolved in organic solvent as ethyl-acetate in order to prevent the lactonolysis (i.e. opening of the HSL ring) that will occur in prolonged exposure to aqueous conditions.

Methods

Preparation of plant extracts with organic solvents such as hexane, chloroform, ethyl acetate, and ethyl alcohol

Some common extraction methods

- Dried or wet, fresh plant parts are ground in a blender to fine particles, put in a certain quantity of solvent and shaken vigorously for 5-10 min or left for 24 h after which the extract is filtered.
- The filtrate then may be dried under reduced pressure and re-dissolved in the solvent to determine the concentration.
- Some researchers however centrifuged (approximately 20,000 × g, for 30 min) the filtrate for clarification of the extract.
- Another common method is serial exhaustive extraction which involves successive extraction with solvents of increasing polarity from a non polar (hexane) to a more polar solvent (methanol) to ensure that a wide polarity range of compound could be extracted.

Gurjar *et al.*,2012

Methods

Extraction

Preparation of water plant extracts

- Dried material was added to sterile water at 100 g dry wt/L, and boiled for 5 min.
- An aliquot was removed for testing.
- The remainder of the decoction was frozen at -80°C for 24 h and then freeze-dried using a lyophilizer.
- The lyophilized extracts remained at -20°C to be reconstituted in sterile water as needed.
- Filtration of the water extracts using a 0.2 µm (pore size) filter into autoclaved vials ensured sterility of the samples.
- Extracts were tested for microbial contamination at every step of processing, by streaking to LB agar plates, to minimize the potential for introduction of exogenous anti-QS compounds.

Adonizio *et al.*,2006

Methods

Preparation of plant extracts with ethanol

- The air-dried plant materials were separately extracted twice at room temperature with ethanol 95% (500 ml/100 g of plant material each run).
- The final ethanol extract of each plant part was filtered using filter paper (Whatman) and evaporated under vacuum at 40°C using a rotary vacuum evaporator in order to remove all the ethanol.
- The resultant residues from the different plants parts were further fractionated according to Mahasneh, 2000 and were stored at -20°C for further analysis.

Al-Hussaini and Mahasneh,2009

Methods

Preparation of plant extracts with ethanol

- Cured vanilla beans (*V. planifolia* Andrews) were shade dried and ground to powder.
- Powdered samples of 100 g were extracted using 600 ml of 75% (v/v) aqueous methanol for 2 day and repeated twice.
- The extract was concentrated (29.96 g) with a rotary evaporator and redissolved in appropriate concentrations of dimethyl sulfoxide (DMSO) for further experiments.

DMSO is an important colorless polar aprotic solvent.

Choo *et al.*,2006

Methods

Preparation of plant extracts with organic solvents such as hexane, chloroform, ethyl acetate, and ethyl alcohol

- All plant species were collected at the flowering stage between May and September.
- The dried plant material was milled to a fine powder.
- The dried powder (10 g) was extracted for 24 hours using hexane, chloroform, ethyl acetate, and ethyl alcohol.
- Solvents of all the extracts were removed under low vacuum by using a rotary evaporator.
- The crude extracts were reconstituted in 10 mL methanol to a final concentration of 10 g/10 ml and sterilized by filtration through a 0.45 µm membrane filter and stored at -20ºC.
- Extracts were tested for microbial contamination at every step of processing by streaking to Luria-Bertani (LB) agar plates.

Bosgelmez *et al.*,2007

Methods

Preparation of plant extracts with organic solvents such as hexane, chloroform, ethyl acetate, and ethyl alcohol

- Fresh thyme (10 g) was cut into ½-inch pieces and allowed to dry in an aerated incubator at 55ºC for 48 hrs.
- The plant material was extracted with 200 mL of ethanol in a covered flask for 24-48 hours.
- Thyme plant material also extracted with solvents including acetone, chloroform, ethyl acetate, hexane, methanol, methylene chloride, and petroleum ether.

Nagy,2010

Methods

Preparation of plant extracts with organic solvents such as hexane, chloroform, ethyl acetate, and ethyl alcohol

Percentage Yield

- The percentage yield of crude plant extracts was calculated as follows:

$$\frac{\text{Weight of crude plant extract x100}}{\text{Weight of starting bulk dried plant material}} = \text{Percentage yield}$$

Nagy,2010

Methods

Plant biomass determination

Calculation of fresh and dry weight of plants

- Plant tissue will be placed in paper bags or aluminum weighing dishes (depending on sample size) in a drying oven set at 80°C.
- The tissue will be dried for 24-48 hours, cooled in a desiccator jar, and reweighed (dry weight).
- The tissue will be weighed at 4 to 8 hour intervals, replacing the material in the oven between weighings, until no more water weight is lost (i.e., to a constant weight).
- Care must be taken not to cook or char the material.
- If oven space is limited, materials can be held refrigerated for no more than one week prior to drying.
- Less succulent tissues may be left to dry at room temperature in open paper bags before completing the process in the oven.
- It is important not to allow the samples to decay before drying.

Nagy,2010

Methods

Preparation of plant extracts with organic solvents such as hexane, chloroform, ethyl acetate, and ethyl alcohol

Crude weight and yield percentage

- Percentage yield of crude extracts produced by extraction of Thyme leaves in various solvents.

$$\frac{\text{Weight of crude plant extract x100}}{\text{Weight of starting bulk dried plant material}} = \text{Percentage yield}$$

Nagy,2010

Methods

Preparation of plant extracts with organic solvents such as hexane, chloroform, ethyl acetate, and ethyl alcohol

Percentage Yield

- The percentage yield of crude plant extracts was calculated as follows:

$$\frac{\text{Weight of crude plant extract x100}}{\text{Weight of starting bulk dried plant material}} = \text{Percentage yield}$$

Nagy,2010

Classification of solvents

Organic solvents

- Oxygenated solvent is an organic solvent, molecules of which contain oxygen.
- Examples: alcohols, glycol ethers, methyl acetate, ethyl acetate, ketones, esters, and glycol ether/esters.
- Chemists have classified solvents into three categories according to their polarity:

1. Polar protic
2. Polar aprotic
3. Non-polar solvents.

Organic solvents

1. Polar protic solvents (hydrophilic solvents):

- Protic describes a molecule that contains an O-H bond.
- Usually polar solvent has O-H bond of which water (HOH), methanol (CH_3OH), and acetic acid (CH_3COOH), ethanol (CH_3CH_2-OH), n-propanol ($CH_3CH_2CH_2$-OH), n-butanol ($CH_3CH_2CH_2CH_2$-OH).

2. Polar aprotic solvents (hydrophilic solvents):

- Aprotic describes a molecule that does not contain an O-H bond but has a C=O bond typically.
- Miscible with water.
- Examples are acetone [$(CH_3)_2C=O$] and ethyl acetate ($CH_3COOCH_2CH_3$).

Organic solvents

3. Non-polar solvents(lipophilic solvents):

- Contain a C=O bond without O-H bond.
- Immiscible with water.
- Non-polar solvents are liphophilic as they dissolve non-polar substances such as oils, fats, greases.
- Example:
- carbon tetrachloride (CCl_4),
- benzene (C_6H_6), and
- diethyl ether ($CH_3CH_2OCH_2CH_3$), hexane (C_6H_{12}), methylene chloride (CH_2Cl_2), toluene(C_7H_8).

Organic solvents

Common solvents for organic reactions

It should be apparent from the table that there are no sharp boundaries between polar and non-polar solvents.

Polar protic solvents	Structure	bp, °C
water	H-OH	100
Methanol	CH_3-OH	68
ethanol	CH_3CH_2-OH	78
1-propanol	$CH_3CH_2CH_2$-OH	97
1-butanol	$CH_3CH_2CH_2CH_2$-OH	118
formic acid	H-C(=O)OH	100
acetic acid	CH_3-C(=O)OH	118
formamide	CH_3NO	210
....	**....**	**....**

Organic solvents

Common solvents for organic reactions Continued

It should be apparent from the table that there are no sharp boundaries between polar and non-polar solvents.

Polar aprotic solvents	Structure	bp, °C
acetone	CH_3-C(=O)-CH_3	56
methyl ethyl ketone	C2H5COCH3	80
ethyl acetate	CH_3-C(=O)-O-CH_2-CH_3	78
acetonitrile	CH_3-C≡N	81
N,N-dimethylformamide (DMF)	H-C(=O)N(CH_3)$_2$	153
diemthyl sulfoxide (DMSO)	CH_3-S(=O)-CH_3	189
Non-polar solvents	**....**	**....**
hexane	$CH_3(CH_2)_4\,CH_3$	69
benzene	C_6H_6	80
diethyl ether	$CH_3CH_2OCH_2CH_3$	35
tetrahydrofuran (THF)	C_4H_8O	66
methylene chloride	CH_2Cl_2	40
carbon tetrachloride	CCl_4	76

Organic solvents

Polarity order

Polarity	Group	Formula
Polar	Water	H-OH
↑	Carboxylic Acids	R-COOH
↑	Amides	$R\text{-}CONH_2$
↑	Alcohols	R-OH
↑	Amines	$R\text{-}NH_2$
↑	Ketones (Aldehydes)	R-CO-R'
↑	Esters	R-COOR'
↑	Alkyl Halides	R-X
↑	Ethers	R-O-R'
↑	Aromatics	Ar-H
Non-polar	Alkanes	R-H

Methods
Preparation of AHL stock solutions
The solvents

- Stock solutions (1 mM) of AHLs compounds such as C4 AHL, C6 AHL, 3oxo-C6 AHL, etc. can be prepared in LB broth or solvents such as DMSO or acetic acid-acidified ethyl acetate (0.01% vol/vol) (BDH) (HPLC grade) and for C14 AHL, it is prepared in dichloromethane (BDH) solvent (HPLC grade).
- The stock QS solutions (AHLs) at the final concentration of 0.12 µg/mL were sterilized by filtration using a 0.22 µm-pore-size filter membrane (Millipore).

AHLs were also prepared by dilution in phosphate-buffered saline, PBS (10 mM, pH 7.4) or 100 µl of 20% or 5mM DMSO.

AL-Ghonaiem *et al.*,2009; Norizan *et al.*,2013;..

Methods

Identification and quantification of AHLs

1. Bioassays: Using biosensors such as *C.violaceum* CV026 which detects AHL-like molecules with short acyl chains and *A. tumefaciens* NTL4 (pZLR4) which detects AHLs with long and short acyl chains.
2. Preparation of AHL Extracts: From bacteria showing AntiQS activity.
3. β-Galactosidase Assay (whenever *A. tumefaciens* NTL4 (pZLR4) was used to detect AHLs). Natures of bacterial AHLs were further studied by quantification of β-galactosidase activity levels.
4. Violacein extraction: used for quantification of Violacein.
5. Thin-Layer Chromatography for AHLs: For detection of QS molecules from bacteria.

Nievas *et al.*,2012;..

Methods
Identification and Quantification of AHLs

6. Thin-Layer Chromatography for crude plant extracts:

- Based on TLC results, it appears that the active component can be extracted in all the solvents tested, but the hexane extract contained the least number of inactive compounds

7. High-Performance Liquid Chromatography and Mass Spectrometry (LC-MS/MS):

- Strains displaying moderate to high levels of AHL-like inducer activity were subjected to chemical identification of signaling molecules by high-performance liquid chromatography coupled to mass spectrometry (LC-MS/MS).

Nievas *et al.*,2012

Methods

Identification and Quantification of AHLs

TLC assays

- Reverse-phase (RP)-TLC plates were used to determine AHL signal profiles.
- Concentrated aEtOAc extracts(bacterial culture supernatants resuspended from PDA plates were extracted with acidified ethyl acetate) were spotted on to the TLC origin in 2-mL volumes and from 0.5- to 2-mL supernatant equivalents were loaded per lane onto a C18 RP-TLC plate.
- Plates were developed in a 60% methanol: water mobile phase, dried and AHLs were detected as described (Scott *et al.*,2006).
- AHL signals were identified with appropriate reference compounds.
- This involves determining and comparing retardation factors (Rf) of unknown samples to AHL reference compounds (McClean *et al.*, 1997; Shaw *et al.*,1997).
- TLC analyses were repeated at least twice.

Lowe *et al.*,2009

Methods

Anti-QS activity of piper plants

Preparation of CV026 suspension

- The bacterial strain, which was kept in Eppendorf tubes at -70°C, was revived in Luria-Bertani agar (LB) and incubated for 18 hours at 30°C.
- After this process, culture colonies were transferred to LB broth; cell density was read at a wavelength of 620 nm, and then adjusted to 0.5 in the Mc-Farland scale.
- This value is equivalent to an absorbance of 0.08-0.1 according to the National Committee for Clinical Laboratory Standards (NCCLS).

CV026 was cultured in Luria-Bertani (LB) agar (1% w/v peptone, 0.5% w/v yeast extract, 0.5% w/v NaCl, agar 1.5% per 100 mL distilled water).

Olivero *et al.*,2011

Methods

Anti-QS activity of piper plants

Preparation of essential oil dilutions

- 100 µL of CV026 suspension were placed in separate tubes containing LB broth and C6-homoserine lactone (HHL); LB broth, C6-homoserine lactone (HHL) and DMSO; and LB broth, HHL and different dilutions of essential oil (tubes A, B and C respectively, as shown in table.
- Essential oils of three species of *Piper* plants were dissolved in DMSO and then added to the culture medium to obtain concentrations of 0.01, 0.1, 1, 10, 50, 100, 200, 300, 400, 500, 750 and 1000 µg/ml.
- The max. amount of DMSO used in the assays was 0.5%.
- DMSO (100%) was used as positive control.

	A	B	C	Final Concentration
LB	895 µL	890 µL	890 µL	-
CV026	100 µL	100 µL	100 µL	1 x 10^8 ufc/ mL
HHL	5 µL	5 µL	5 µL	15 µmol/mL
OIL	-	-	5 µL	(Variable)
DMSO	-	5 µL	-	
Total Volume	1 mL	1 mL	1 mL	

Composition of the tubes for determining antiquorum sensing activity

Olivero *et al.*,2011;..

Methods

Anti-QS activity in piper

CV026 cell growth in presence of different concentrations of Piper EOs

- Cell growth of *Chromobacterium violaceum* CV026 after exposure different concentrations of EOs from Piper spp.
- Bacterial growth remained greater than 50% even at concentrations greater than 1000 µg/ml.

[EO] (µg/mL)	*P. bredemeyeri*	*P. brachypodom*	*P. bogotense*
Control	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
0.01	99.1 ± 0.2	92.7 ± 1.3	99.0 ± 0.3
0.1	96.6 ± 0.4	91.9 ± 1.5	96.8 ± 0.6
1	93.7 ± 0.5	90.8 ± 1.8	94.0 ± 0.5
10	93.1 ± 0.4	90.3 ± 1.3	93.4 ± 0.6
50	91.7 ± 0.5	89.5 ± 1.0	92.0 ± 0.4
100	88.4 ± 0.4*	88.0 ± 1.5*	89.0 ± 0.4*
200	84.5 ± 0.6*	86.5 ± 0.9*	87.6 ± 0.6*
300	82.4 ± 0.5*	85.9 ± 1.2*	84.6 ± 0.2*
400	80.4 ± 0.5*	83.5 ± 1.5*	83.3 ± 0.5*
500	78.7 ± 0.4*	84.2 ± 0.8*	79.0 ± 0.4*
750	70.7 ± 0.4*	87.0 ± 0.9*	78.1 ± 0.3*
1000	67.0 ± 0.5*	86.3 ± 1.0*	77.0 ± 0.4*
IC_{50}	> 1000	> 1000	> 1000

* Significant differences when compared to control vehicle-treated cells (ANOVA; Dunn's test)

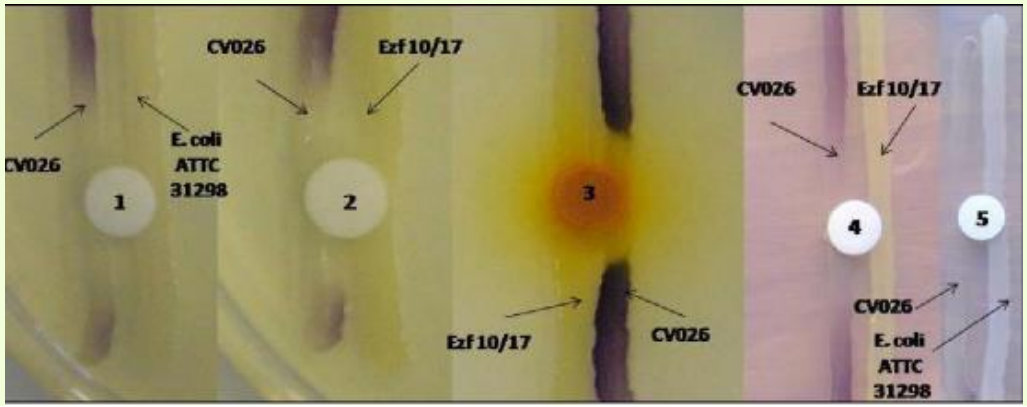
Olivero *et al.*,2011

Methods

Anti-QS activity in piper

CV026 cell growth in presence of Geranium and rose oils

- Inhibition of QS by agar diffusion method. The interference of essential oils with the autoinducer production of *E. coli* 31298 and *Chromobacterium violaceum* CV026.
- Geranium oil was most effective and also Ezf 10/17 rose oil inhibit violacein production (discs 4 and 5).
- Discs 1, 2, and 3(5-FU, 5-FU and AO are positive controls,
- Discs 4, rose oil and 5, Geranium oil.



Schelz,2007

Methods

Antiquorum sensing assays

The density of *Chromobacterium violaceum* CV026

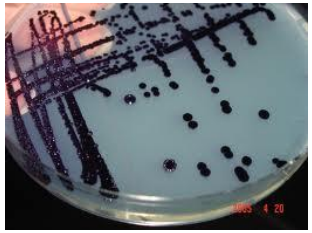
- *Chromobacterium violaceum* CV026 was incubated for 16-18 h, and 100 µl of the bacteria, adjusted to OD_{600nm} = 0.1 (approximately 1 × 10^8 CFU ml^{-1}), were spread on agar plates.
- Or
- Cell density was read at a wavelength of 620 nm, and then adjusted to 0.5 Mc-Farland scale. This value is equivalent to an absorbance of 0.08-0.1 according to the National Committee for Clinical Laboratory Standards (NCCLS,2006)

Choo *et al.*,2006; Olivero *et al.*,2011

Methods

Chromobacterium violaceum

Colony morphology



- It is a Gram-negative, facultative anaerobic, non-sporing coccobacillus.
- It is part of the normal flora of water and soil of tropical and sub-tropical regions of the world.
- It produces a natural antibiotic called violacein.
- It grows readily on nutrient agar, producing distinctive smooth, low convex, circular, with an entire margin colonies with a dark violet metallic sheen (due to violacein production).

Methods

Biomonitor/Bioreporter/Biosensor or reporter strains

- These biosensors do not possess the ability to produce any AHLs.
- Biosensors such as *Escherichia coli* (pSB401), *E. coli* (pSB1075), *Chromobacterium violaceum* CV026 are used by researchers to aid the screening for compounds/extracts with anti-QS abilities.
- External AHLs are supplied exogenously to induce QS traits such as bioluminescence and violacein production which can be quantified.
- The anti-QS ability of compounds/extracts are measured by the significance of the inhibition.

Koh *et al.*,2013

Methods

Biomonitor/Bioreporter/Biosensor or reporter strains

1. ***Chromobacterium violaceum*** (a biosensor sensitive to (activated by) short-chain AHLs):

- The wild type *C. violaceum* (ATCC 12472) produces purple pigment violacein when it was exposed to the cognate autoinducer molecules C6-AHL and C4-AHL.

2. **The non-pigmented mutant strain, *C. violaceum* CV026** (NCTC13278):

- *C. violaceum* CV026 is an AHL biosensor that will produce a purple pigment in the presence of short chain AHL.
- *C. violaceum* CV026 lacks the autoinducer synthase CviI that controls the synthesis of violacein and thus requires exogenous C6-HSL for violacein formation, which is QS-mediated.

Methods

Biomonitor/Bioreporter/Biosensor or reporter strains

Broad-spectrum AHL TraR-dependent *A. tumefaciens* NTL4 (pZLR4) biosensor

3. ***Agrobacterium tumefaciens*** **NTL4 (pZLR4) ATCC® BAA-2240™ :** *A. tumefaciens* strain NTL4 containing plasmid pZLR4 that carries a *traG::lacZ* reporter fusion and *traR*.

- In the presence of AHLs with long acyl chains the TraR protein is activated, transcription of the *traG::lacZ* fusion is turned on, and LacZ (β-galactosidase) activity can be used as a reporter of *traG* transcription.
- *A. tumefaciens* reporter strain NT1 detects a broad range of AHSLs (good sensitivity on TLC) and was used for the detection of both short (C6) and long acyl chains (C12) AHL suppression.
- Anti-QS compounds inhibit the QS-dependent promoter and subsequent *lacZ* expression, thus limiting X-gal hydrolysis, and the appearance of blue color.

Adonizio,2008; González *et al.*,2009; Koh *et al.*,2013

Methods

Biomonitor/Bioreporter/Biosensor or reporter strains

4. ***P. aeruginosa*** **PAO1(ATCC 27853):** A wild type strain of *P. aeruginosa* which is used for anti-swarming bioassays.
5. ***Escherichia coli*** **(pSB401);**
6. ***E. coli*** **(pSB1075);**
7. ***P. putida*** **F117(pAS-C8);**
8. ***Serratia liquefaciens*** **MG44(pJBA132).**

Please note that different biosensor strains of *E. coli* respond to a range of different AHLs. E.g.
-*E. coli* (pSB401) produces bioluminescence in the presence of short chain AHL.
-The strain *E. coli* (pSB403) is able to respond to a range of different AHLs by luciferase production (e.g., BHL, HHL, OHHL, and ODHL).
-Biosensors *E. coli* JM109 [pSB1075] and *E. coli* JM109 [pSB536] for detection of Long(C12) and short chain AHLs(C4), respectively.

Steidle *et al.*,2001;Adonizio,2008; Koh *et al.*,2013;..

Methods

Biomonitor/Bioreporter/Biosensor or reporter strains

- *Chromobacterium violaceum* ATCC 12472 and C. violaceum CV026, purchased from the American Type Culture Collection (ATCC).
- In *C. violaceum* ATCC 12472, production of the natural antibiotic violacein, a water-insoluble purple pigment with antibacterial activity, is under the control of a QS system.

It should be noted that CV026 does not respond to long chain AHLs and AHL production (N-acyl side chains C4-C8) was judged by its ability to induce violacein production.

Methods

Biomonitor/Bioreporter/Biosensor or reporter strains

Chromobacterium violaceum CV026

Strain/Plasmid	Host	Based on QS system	Reporter system	Best responds to	Good detection	Commonly used for
C. violaceum CV026	*C. violaceum*	CviI/R (*C. violaceum*)	Violacein pigment	C6-AHL	C6-3-oxo-AHL C8-AHL C8-3-oxo-AHL C4-AHL	T.S., TLC

*T.S. refers to 'T' streak analysis in solid media.
Note that TLC analysis of unusually long AHLs results in no migration.

Steindler and Venturi,2007

Methods

Biomonitor/Bioreporter/Biosensor or reporter strains

Agrobacterium tumefaciens NTL4 (pZLR4)

Strain/Plasmid	Host	Based on QS system	Reporter system	Best responds to	Good detection	Commonly used for
pZLR4	*A. tumefaciens* NT1	*tume*TraI/R (*A. faciens*)	β-galactosidase	C8-3-oxo-AHL	All 3-oxo-AHLs C6-AHL C8-AHL C10-AHL C12-AHL C14-AHL C6-3-hydroxy-AHL C8-3-hydroxy-AHL C10-3-hydroxy-AHL	T.S., TLC, Q.

T.S. refers to 'T' streak analysis in solid media.
Note that TLC analysis of unusually long AHLs results in no migration.
Q refers to quantification.
Quorum sensing uses a low molecular weight chemical signal (almost all belonging to the chemical family called N-oxoacylhomoserine lactones (Szenthe and Page,2003). The main 3-oxo-AHLs are: C6-3-oxo-AHL, C8-3-oxo-AHL; C10-3-oxo-AHL, C12-3-oxo-AHL, C14-3-oxo-AHL, C16:1-3-oxo-AHL.

A. tumefaciens NT1 (or NTL4) is a broad-spectrum AHL TraR-dependent biosensor. HSLs with acyl-chain length of C6 to C12 are readily detected by the indicator strain. NTL4 (pZLR4) responds preferentially to 3-oxo-C8-HSL and to unsubstituted and 3-oxo signals, except for C4-HSL.

Steindler and Venturi,2007;..

Methods

Biomonitor/Bioreporter/Biosensor or reporter strains

Biosensor systems using

Overview of plasmid-based AHL reporter bacteria

Plasmid	Bacterial strain	Analyte	Promoter	Reporter
pSB401	*E. coli*	3-oxo-C4- to 3-oxo-C14-HSL C4- to C12-HSL	*luxI*	*luxCDABE*
pSB1075	*E. coli*	3-oxo-C12- to 3-oxo-C16-HSL C12- to C16-HSL	*lasI*	*luxCDABE*
pECP61.5	*P. aeruginosa*	C4-HSL	*rhlA*	*lacZ*
pKDT17	*E. coli*	3-oxo-C10- to 3-oxo-C12-HSL C10- to C12-HSL	*lasB*	*lacZ*
pCF218, pMV26	*A.tumefaciens*	3-oxo-C6- to 3-oxo-C12-HSL C4- to C12-HSL	*traI*	*luxCDABE*
pCF218, pCF372	*A.tumefaciens*	3-oxo-C4- to 3-oxo-C12-HSL C5- to C10-HSL	*traI*	*lacZ*
pSB406	*E. coli*	3-oxo-C4- to 3-oxo-C14-HSL C4- to C12-HSL	*rhlI*	*luxCDABE*
	C. violaceum	3-oxo-C6- to 3-oxo-C8-HSL C4- to C8-HSL	*cviI*	Violacein
pAL105	*E. coli*	3-oxo-C12-HSL	*lasI*	*luxCDABE*
pAL101	*E. coli*	C4-HSL	*rhlI*	*luxCDABE*
pSB536	*E. coli*	C4-HSL	*ahyI*	*luxCDABE*
pSB403	*Broad host range*	3-oxo-C4- to 3-oxo-C14-HSL C4- to C12-HSL	*luxI*	*luxCDABE*
pHV2001	*E. coli*	3-oxo-C6- to 3-oxo-C8-HSL C6- to C8-HSL	*luxI*	*luxCDABE*
pZLR4	*A.tumefaciens*	All 3-oxo-HSLs C6- to C14-HSL 3-OH-C6- to 3-OH-C10-HSL	*traI*	*lacZ*
pJZ384, pJZ410, pJZ372	*A.tumefaciens*	3-oxo-C4- to 3-oxo-C18-HSL C4- to C18-HSL	*traI*	*lacZ*
pSF105, pSF107	*P. fluorescens*	3-OH-C6-HSL C6-HSL 3-OH-C8-HSL	*phzI*	*lacZ*
pUCP18	*P. aeruginosa*	3-oxo-C12-HSL	*rsaL*	*luxCDABE*
pMS402	*P. aeruginosa*	3-oxo-C12-HSL	*rsaL*	*luxCDABE*
pUCGMAT1-4	*E. coli*	3-oxo-C6-HSL	*ahlI*	*mcherry*
pREC-FF	*E. coli*	3-oxo-C6-HSL	*luxI*	*cfp*

Verbeke *et al.*,2017

Evaluation of the synergistic/antagonistic activities of two compounds

Fractional Inhibitory Concentration Index (FIC Index) calculations

- **Interpretation:**

1. Synergy= mean FICI ≤ 0.5.
2. Partial synergy/addition= mean FICI >0.5 ≤ 1.0.
3. Indifference= mean FICI >1 - <2.0.
4. Antagonism= mean FICI ≥ 2.0.

- *FIC: Fractional inhibitory concentration.
- **FICI: Fractional inhibitory concentration index.

Formula to determine synergy

$$FIC^*_A = \frac{MIC_A \text{ in combination}}{MIC_A} \qquad FIC_B = \frac{MIC_B \text{ in combination}}{MIC_B}$$

Where A and B are the two antimicrobials under investigation

$$FICI = FIC_A + FIC_B$$

$$\text{Mean FICI}^{**} = \frac{\text{Sum of FICI calculated}}{\text{Number of FICI calculated}}$$

The same formula in different notation

$$FIC\ (A) = \frac{MIC\ A\ (in\ the\ presrnce\ of\ B)}{MIC\ A}$$

$$FIC\ (B) = \frac{MIC\ A\ (in\ the\ presrnce\ of\ B)}{MIC\ A}$$

$$FIC\ (index) = FIC\ (A) + FIC\ (B)$$

Bharadwaj *et al.*,2003;..

Evaluation of the synergistic/antagonistic activities of two compounds

Fractional Inhibitory Concentration Index (FIC Index) calculations

- Synergy: Synergistic action of a combination of antibiotics is present if the effect of the combination exceeds the additive effects of the individual components.
- Partial synergy/addition: The additive effect of combination is one in which the effect of combination is equal to that of the sum of the effects of the individual components.
- Indifference: An indifferent effect of a combination is one that is equal to the effects of the most active component.
- Antagonism: Antagonism is present if a reduced effect of a combination of antibiotics is observed in comparison with the effect of the most effective individual substance.

Bharadwaj *et al.*,2003

Evaluation of the synergistic/antagonistic activities of two compounds

Fractional Inhibitory Concentration Index (FIC Index) calculations

- MIC_A MIC of antibiotic A alone;
- MIC_B MIC of antibiotic B alone;
- MIC_{AB} MIC of A in the presence of antibiotic B;
- MIC_{BA} MIC of B in the presence of antibiotic A.
- Fractional Inhibitory Concentration Index (FIC Index) calculations:
- FIC Index = $MIC_{AB}/MIC_A + MIC_{BA}/MIC_B$.

Interpretation FIC	Examples
Synergy ≤ 0.5 Additative > 0.5 and ≤ 1.0 Indifference > 1 and ≤ 4.0 Antagonism > 4.0	(2/8 + 2/16) = 0.25 + 0.125 = 0.375 - (1/1 + 1/2) = 1 + 0.5 = 1.5 (16/4 + 16/16) = 4+1 =5

AB BIODISK,2007

Evaluation of the synergistic/antagonistic activities of two compounds

Fractional Inhibitory Concentration Index (FIC Index) calculations

- Effect of norfloxacin and metronidazole combination on aerobic isolates.

Isolates	Source	Number of isolates				
		S	PS	NE	A	Total
Escherichia coli	Urine	03	03	0	0	06
Klebsiella spp	Urine	0	06	0	0	06
Pseudomonas spp	Urine	0	06	0	0	06
Salmonella spp	Stool	01	05	0	0	06
Shigella spp	Stool	0	06	0	0	06
Proteus spp	Urine	02	04	0	0	06

S: Synergy, mean FICI ≤ 0.5; PS: Partial synergy, mean FICI >0.5 ≤ 1.0; NE: No effect; A: Antagonism, mean FICI ≥ 2.0.

According to Odds,2003, interpretations of synergy (FICI ≤ 0.5), antagonism (FICI > 4.0) and no interaction(FICI > 0.5-4.0).

Bharadwaj *et al.*,2003;Odds,2003

Methods

2. Antiquorum sensing assays

- Fresh or dried plant material was ground using a mortar and pestle and placed directly onto Luria Bertani (LB) plates spread with either:

1. *C. violaceum* (wt),
2. CV026 supplemented with AHL, or
3. *A. tumefaciens* NTL4 supplemented with AHLs and X-gal.

- *Chromobacterium violaceum* cultures were maintained at room temperature for daily use.
- Stock cultures were kept at -20°C in Luria Bertani broth (4 mL) supplemented with 25% (v/v) sterile glycerol as a cryoprotectant.

Adonizio,2008; Al-Hussaini and Mahasneh,2009

Methods

Antiquorum sensing assays

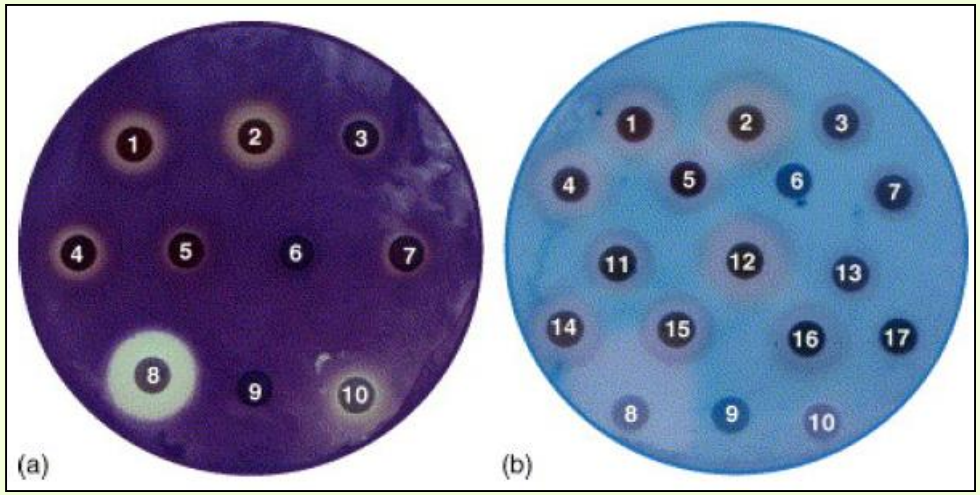
- Violacein production is a phenotype that is regulated by QS in *C. violaceum*.
- Plant and microbial extracts with anti-QS properties inhibit violacein production, a QS-regulated behavior in *Chromobacterium violaceum*.
- Lack of purple pigmentation from *C. violaceum* in the vicinity of the test extracts indicated the inhibitory effect of the plant extracts.
- The growth curves of *C. violaceum* showed either activities of some extracts:

1. *C. violaceum* growth inhibition, or
2. Inhibition of *C. violaceum* violacein production.

Anti-QS activity

Using *C. violaceum* (a) and *A. tumefaciens* strain (b)

- Anti QS activity was tested using 20 µL of water extracts of (1) *C. erectus*, (2) *B. buceras*, (3) *C. viminalis*-leaf, (4) *C. viminalis*-infloresence, (5) *T. bicolor*, (6) *C. hypericifolia*, and (7) *Q. virginiana* (7).
- Also included as controls were (8) 10 µg gentamycin (a) and tetracycline (b), (9) 20 µl ethanol, and (10) halogenated furanone.
- Discs 11-17 contain 20 µL of ethanolic extracts of (11) *C. erectus*, (12) *B. buceras*, (13) *C. viminalis*-leaf, (14) *C. viminalis*-infloresence,(15)*T. bicolor*, (16) *C. hypericifolia*, and (17) *Q. virginiana*.



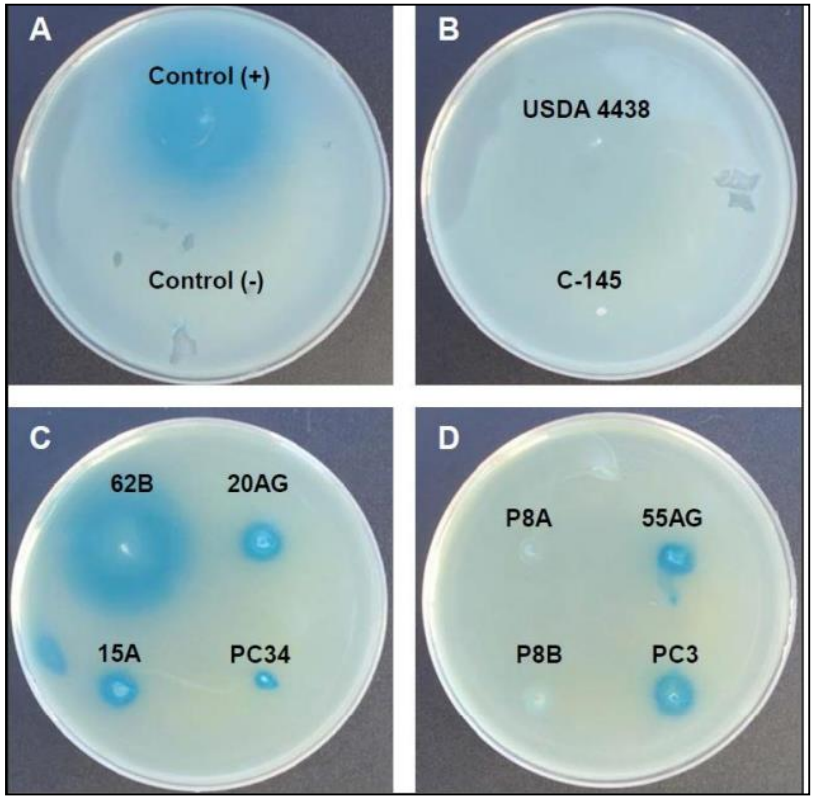
C. violaceum *A. tumefaciens*

Adonizio,2008

Anti-QS activity

Bioassay for detection of AHL-like molecules with long acyl chains using *A. tumefaciens* NTL4 (pZLR4) as biosensor strain

- Plate A: Control(+): with *A. tumefaciens* NTL4 and Control(-) without *A. tumefaciens* NTL.
- Plate B, C, D: Nodulating bacterial strains.
- Plate B: Non-AHL producing bacterial strains (USDA 4438 and C-145 with no blue haloes).
- Plates C and D: Bacterial strains producing AHL-like molecules with long acyl chains (62B, 20AG, 15A, PC34 55AG, and PC3).
- P8A and P8B showed undefined results (D) for AHL production.



Nievas *et al.*,2012

Anti-QS activity

Bioassay for detection of AHL-like molecules with long acyl chains using *A. tumefaciens* NTL4 (pZLR4) as biosensor strain

- β-Galactosidase Assay:
- The production of AHLs was quantified by measuring β-galactosidase activity using *A. tumefaciens* NTL4 (pZLR4).
- Aliquots (100 µL) of AHL extracts from bacteria were added to 10 mL cultures of *A. tumefaciens* NTL4 (pZLR4) grown to OD_{600} 0.5.
- The cultures were incubated for 6-8 h until reaching OD_{600} 1.0.
- β-galactosidase activity was determined in Miller units as described by Miller,1972.
- For each strain extract, the values presented are means of four repeated experiments.

Nievas *et al.*,2012

Anti-QS activity

Bioassay for QSI using *C. violaceum*

- 5 ml of molten Soft Top Agar (STA) (1.3 g agar, 2.0 g tryptone, 1.0 g sodium chloride, 200 mL deionised water) were seeded with 100 µL of an overnight LB culture of CV026, together with 20 µL of 100 µg /mL C6HSL as an exogenous AHL source.
- This was gently mixed and poured immediately over the surface of a solidified LBA plate as an overlay.
- Wells of 5 mm in diameter were made on the solidified agar of each plate. Each well was filled with 50 µL of filter-sterilised TCM extract.
- A positive control well contained 10 µL of 100 µg/mL C10HSL (*N*-decanoyl-L-homoserine actone DHL, a reported antagonist of violacein synthesis (McLean *et al.*,2004) and 40 µL LB broth.

TCM: Traditional Chinese medicine plants

Yeo and Tham,2011

Anti-QS activity

Bioassay for QSI using *C. violaceum* Continued

- Plates were incubated at room temperature (25 ± 2ºC) for 3 days.
- QS inhibition of violacein synthesis was detected by a turbid or creamy ring of viable cells around the well against a purple background of activated CV026 bacteria (Fig. 1).
- A clear halo around the well would indicate antimicrobial (AM) activity.
- The limit of detection of activity was also determined by serial dilutions of extracts (1:1 to 1:64), using LB broth as diluent (Fig. 2).
- Endpoints were estimated as the lowest dilution of the extract giving visible inhibition of violacein production.
- Each experiment was carried out in triplicate and all assays were repeated twice, except for dilution tests which were repeated once.

TCM: Traditional Chinese medicine plants

Yeo and Tham,2011

Anti-QS activity

Bioassay for QSI using *C. violaceum*

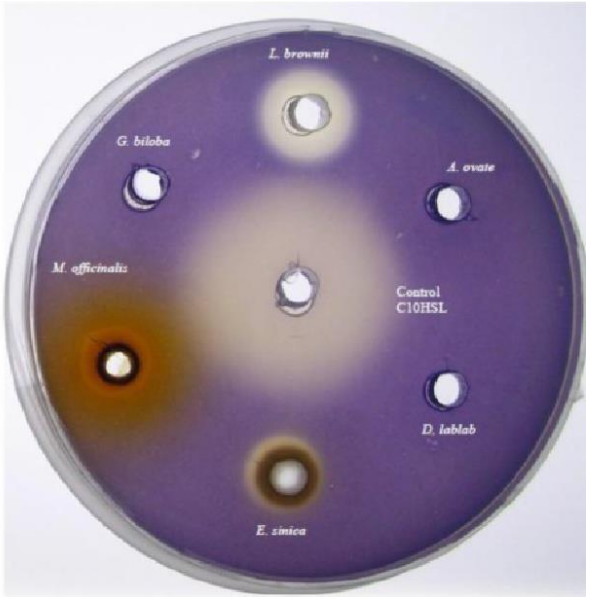


Fig. 1. Bioassay using *Chromobacterium violaceum* CV026 bioreporter strain for testing extracts from traditional Chinese medicinal herbs.
The extracts were from *Lilium brownii*, *Gingko biloba*, *Magnolia officinalis*, *Ephedra sinica*, *Dolichos labab* and *Atractylis ovate*.
A turbid halo indicated an anti-QS effect and a clear halo indicated an antibacterial effect.

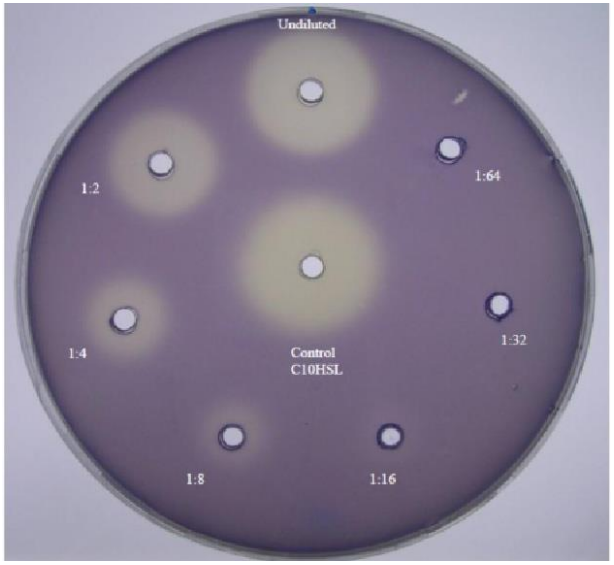


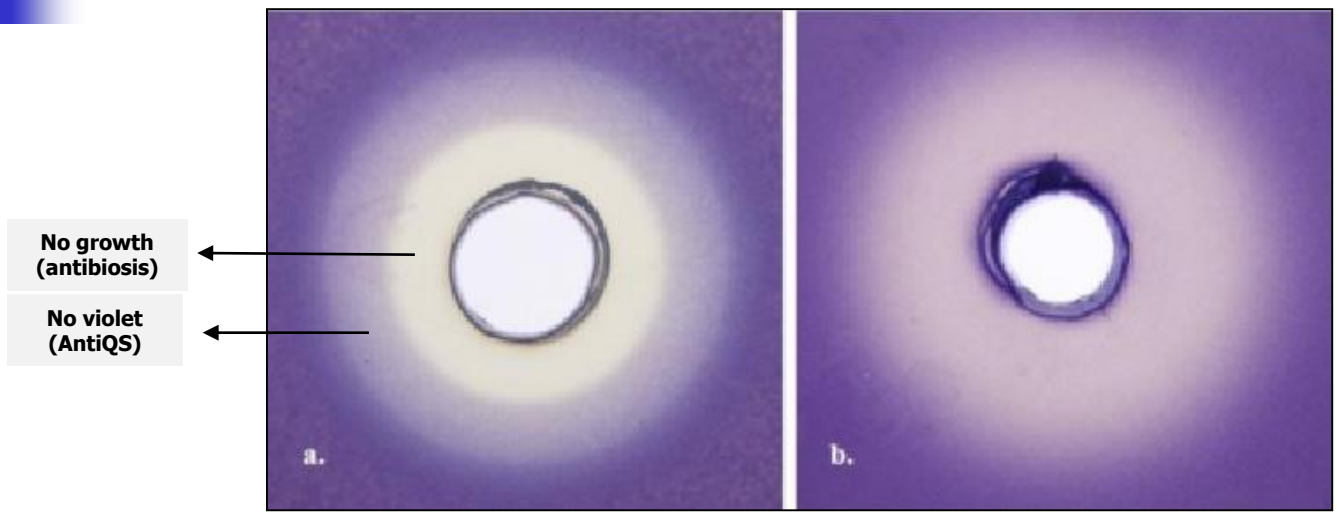
Fig. 2. The TCM herb, *Astragalus membranaceus* (huang qi) showing dilution-dependent quorum-sensing inhibition on *Chromobacterium violaceum* CV026.
Dilutions of 1:64 were carried out and the lowest dilution of *A. membranaceus* showing visibly detectable inhibition of violacein production was 1:16.

TCM: Traditional Chinese medicine plants

Yeo and Tham,2011

Anti-QS activity

Bioassay for both anti-qouorum-sensing and antibacterial sensing activities



Bioassay of TCM extracts using *Chromobacterium violaceum* CV026 showed both anti-quorum sensing and antimicrobial activities.

a. *Lilium brownii* (bai he) extract showing different active principles, i.e.

- antibacterial activity (inner clear ring), and
- QSI or quorum-sensing inhibition (outer creamy ring).

b. *Panax pseudoginseng* (ren shen) extract exhibited only QSI (creamy ring) and no antibacterial activity.

TCM: Traditional Chinese medicine plants

Yeo and Tham,2011

Methods

Preliminary and advanced phytochemicals analyses of plant extracts

- All plant specimens including medicinal plants/mushrooms may contain phenols, flavonoids, quinones, saponins, cardinolites, steroids, tanins and terpenoids in various extracts.

1. Thin layer chromatographic analysis (TLC) is used to find the presence of number of chemical constituents to support the chemical test.
2. Different R_f values were shown the presence of certain phytochemicals in different solvent extracts.
3. After quantitative analysis by TLC, the extracts were further analyzed by HPTLC,GC-MS, HPLC, etc. for better resolution and detection of a wide variety of compounds.

Vindo *et al.*,2009; Ganatra *et al.*,2012;Vigi *et al.*,2013;..

Methods

Phytochemical analysis from Wild Edible Mushroom of *Pleurotus ostreatus Agaricus bisporus*

- **Test for anthraquinones:**
- Weighed mushroom powder, 0.5 g, was boiled in 10% hydrochloric acid and filtered hot. To this, 2 ml chloroform and 10% ammonia solution each were added.
- Formation of pink color in the aqueous layer indicated presence of anthraquinones.
- Anthraquinone, also called anthracenedione or dioxoanthracene, is an aromatic organic compound with formula $C_{14}H_8O_2$. Several isomers are possible, each of which can be viewed as a quinone derivative.

Srividhya *et al.*,2015; Wikipedia,2016

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Srividhya *et al.*,2015; Wikipedia,2016

Methods

Phytochemical analysis from Wild Edible Mushroom of *Pleurotus ostreatus Agaricus bisporus*

- Thin layer chromatographic profiles yielded the different pattern of compound and as well as different Rf values.
- The extracted bioactive compounds were tested followed by calculate their Rf value by analyzing thin layer chromatographic techniques with two different kinds of solvent systems.
- The number of bands and Rf values of each extracts and in suitable solvent systems were presented in next Table.

$$R_f \text{ value} = \frac{\text{Distance travelled by solute from the base line}}{\text{Distance travelled by solvent front from the base line}}$$

Srividhya *et al.*,2015

Methods

Phytochemical analysis from Wild Edible Mushroom of *Pleurotus ostreatus Agaricus bisporus*

1. The ethanol extract showed two compounds in *P. ostreatus* and *A. bisporus* (Rf = 0.36, 0.84) (Rf = 0.40, 0.65) followed by one compound in ethyl acetate (0.63); (0.66), chloroform extract (0.74); (0.63) in Benzene:Ethanol:Acetone (6:2:2) solvent system, respectively.
2. Similarly the other solvent system such as Benzene:Ethanol:Acetone (9:1:0.5) the ethanol extract showed two compounds (Rf = 0.44, 0.64); (Rf = 0.40, 0.78), ethyl acetate extracts (0.64, 0.72); (0.68, 0.79) and two compounds yielded in chloroform extracts (0.34, 0.55) (0.25, 0.60) in *P. ostreatus* and *A. bisporus*, respectively.

Srividhya *et al.*,2015

Methods

Phytochemical analysis from Wild Edible Mushroom of *Pleurotus ostreatus Agaricus bisporus*

Solvent system	*Pleurotus ostreatus*			*Agaricus bisporus*		
	Plant extract	**No. of bands**	**Rf value**	**Plant extract**	**No. of bands**	**Rf value**
Benzene:Ethanol:Acetone (6:2:2)	Chloroform	1	0.74	Chloroform	1	0.63
	Ethyl Acetate	1	0.63	Ethyl Acetate	1	0.66
	Ethanol	2	0.36	Ethanol	2	0.40
			0.84			0.65
Benzene:Ethanol:Acetone (9:1:0.1)	Chloroform	2	0.34	Chloroform	2	0.25
			0.55			0.60
	Ethyl Acetate	2	0.64	Ethyl Acetate	2	0.68
			0.72			0.79
	Ethanol	2	0.44	Ethanol	2	0.40
			0.64			0.78

Srividhya *et al.*,2015

Methods

Preliminary and advanced phytochemicals analyses of plant extracts

- TLC analysis of plant extracts. R_f values of various extracts of *Ficus racemosa* leaves.

Plant Extract	R_f Values *Toluene: Ethyl acetate: Formic acid* Ratio 5 : 1.5 : 0.5
Ethanol	0.85
Methanol	0.57
Ethyl acetate	0.88 0.73 0.54
Acetone	0.97 0.84 0.79
n-Hexane	0.74

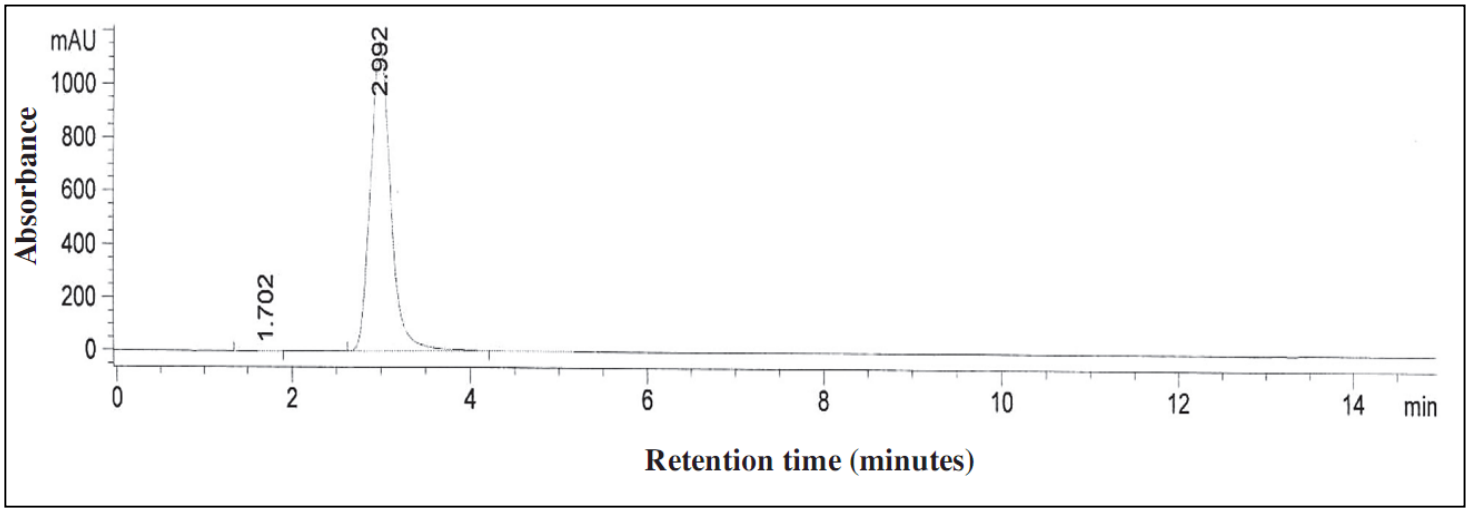
Ganatra *et al.*,2012

Methods

Further analyses

Preparative HPLC Chromatogram obtained for AM3b fraction showing single peak at 2.992 min

- Ethanol fraction was selected and purified active subfractions (AM1, AM2, AM3, AM4, AM5, AM6 and AM7) were characterized by comparing their absorption spectra with that of the known natural products isolated from the plants of *Quercus* genus.



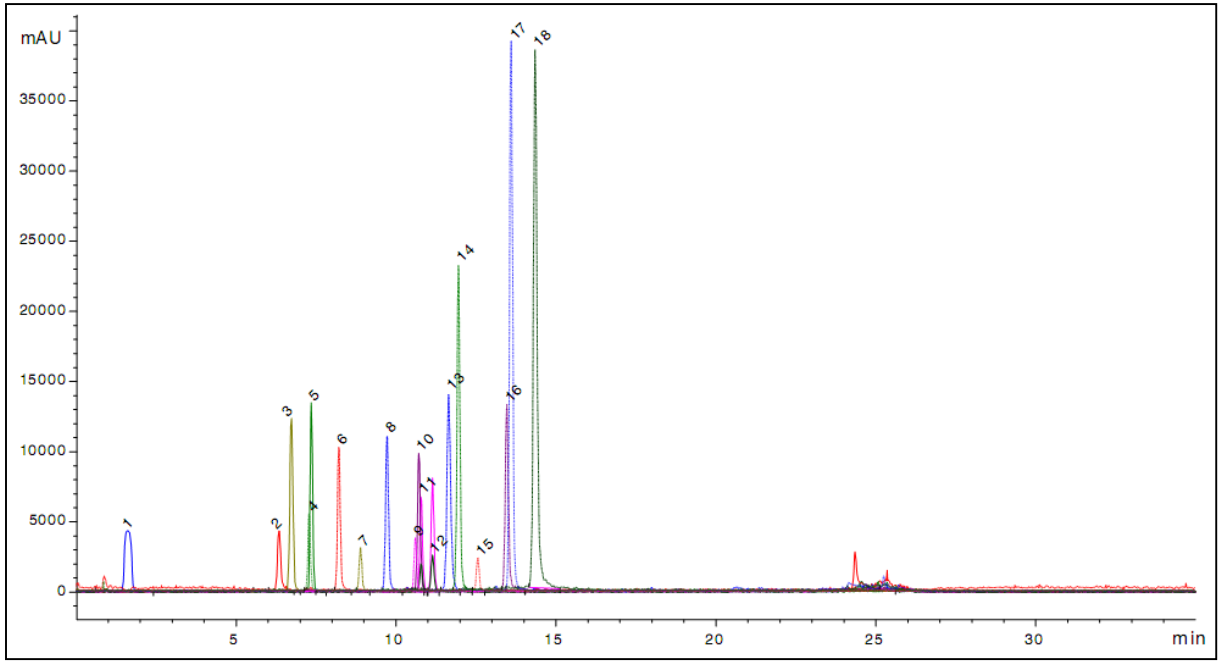
Jamil *et al.*,2012

Methods

Further analyses

Phenolic and flavonoid compounds were characterized by liquid chromatography –mass spectrometry

- ESI-TIC-SIM chromatogram of standard phenolics.
- Analyses were performed with an Agilent Liquid chromatography–mass spectrometry (LC-MS) system (1200 LC with a single quadrupole) with Electrospray Ionisation (ESI) source-negative mode.



Askun *et al.*,2013

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- The extract showed presence of:

1. Carbohydrates,
2. Sterols,
3. Glycosides,
4. Flavonoids, and
5. Phenolic compounds.

Chemical Constituents	Test
Carbohydrates	Molisch test
	Benedicts Test
Glycosides	Borntrager's Test
	Legal's Test
	Keller- Killiani Test
Steroids	Salkowski Test
	Liebermann's Reaction
Tannins & Phenolic Compounds	$FeCl_3$ Sol. Test
	Lead Acetate Test
	Dil. HNO_3 Test
Flavanoids	Shinoda Test
	Lead acetate Test
	Sodium Hydroxide Test
Saponins	Foam Test
Triterpenes	Chloroform + Conc. H_2SO_4

Vindo *et al.*,2009

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- Phytochemicals analysis of different extracts of *Ficus racemosa* leaves.
- The series of solvents in systematic order will help in understanding the effect of polarity on the extraction and extracted Phytochemicals.

Phytochemicals	Extract				
	Ethanol	Methanol	Ethyl acetate	Acetone	n-Hexane
Phenol	+	+	+	+	+
Flavonoid	+	+	+	+	+
Quinones	+	+	-	+	+
Steroids	+	+	+	+	-
Tannins	+	+	-	-	-
Cardiac glycosides	+	+	-	-	-
Terpenoids	-	-	+	-	-
Saponins	-	+	-	-	-
Note : (+) = Present and (-) = Absent					

Ganatra *et al.*,2012

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Tests for carbohydrates:**
- **Fehling's Test:** 1 ml Fehling's A solution and 1 ml of Fehling's B solution were mixed and boiled for one minute.
- Now the equal volume of test solution (ethanolic extract) was added to the above mixture.
- The solution was heated in boiling water bath for 5-10 minutes.
- First a yellow, then brick red precipitate was observed.
- **Benedict's test:** Equal volumes of Benedict's reagent and test solution (ethanolic extract) were mixed in a test tube.
- The mixture was heated in boiling water bath for 5 minutes.
- Solution appeared green showing the presence of reducing sugar.

Vindo *et al.*,2009

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Xanthoproteic Test:**
- Xanthoprotein is a yellow acid substance formed by the action of hot nitric acid on albuminous or protein matter and is changed to a deep orange-yellow colour by the addition of ammonia.
- The colour reaction is used to identify such proteins in the Xanthoproteic Test.

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Xanthoproteic Test(continued):**
- To the small quantity of ethanolic extract 1ml of conc. H_2SO_4 was added.
- This resulted in the formation of white precipitate which on boiling turned yellow.
- On addition of NH_4OH, yellow ppt. turned orange.
- **Biuret Test:**
- Small quantity of ethanolic extract was dissolved in a few mL of water.
- To this test solution 4% NaOH solution and a few drops of 1% $CuSO_4$ solution was added.
- Appearance of violet colour showed presence of proteins.

Vindo *et al.*,2009

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Tests for glycosides:**
- Free content of the sugar extract was determined.
- A class of molecules in which, a sugar molecule is bonded to a "non-sugar" molecule.
- Salicin is an example of an alcholic glycoside. Salicin is found in the genus of plants known as *Salix*.
- The sample was hydrolysed with mineral acid (dilute hydrochloric or dilute sulphuric acid). Again the total sugar content of the hydrolysed extract was determined.
- Increase in the sugar content indicated the presence of glycoside in the extract.

Glycoside ----H_2O ⟶ Aglycon (genin) + Glycon (sugar)

Vindo *et al.*,2009; Srividhya *et al.*,2015

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Tests for glycosides:**
- **Borntrager's Test:** To the 3ml of ethanolic extract, dil. H_2SO_4 was added. The solution was then boiled and filtered. The filtrate was cooled and to it equal volume of benzene was added. The solution was shaken well and the organic layer was separated. Equal volume of dilute ammonia solution was added to the organic layer.
- The ammonical layer turned pink showing the presence of glycosides.
- **Baljet's Test:** To 5 ml of the extract few drops of sodium picrate was added to observe yellow to orange colour.

Vindo *et al.*,2009; Srividhya *et al.*,2015

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Tests for glycosides:**
- **Legal's Test:** To the concentrated ethanolic extract few drops of 10% NaOH were added, to make it alkaline. Then freshly prepared sodium nitroprusside was added to the solution.
- Presence of blue coloration indicated the presence of glycosides in the extract.
- **Keller-Killiani Test:** To 2 ml of the extract, glacial acetic acid, one drop 5% $FeCl_3$ and conc. H_2SO_4 was added.
- Reddish brown colour appeared at junction of two liquid layers and upper layer turned bluish green indicating the presence of glycosides.

Vindo *et al.*,2009;Jamil *et al.*,2012

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Test for saponins:**
- Saponin means "soap." Saponin glycosides form a "soapy froth" when combined with water and shaken.
- They are found in many plants such as oats and spinach.
- **Foam Test:** To 1 ml of the extracts 5 ml distilled water was added and shaken vigorously. Formation of foam indicated presence of saponins.

Vindo *et al.*,2009; Srividhya *et al.*,2015

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Test for terpenoids:**
- **Salkowski's test:** Sample (2 ml) was mixed 2 ml of concentration sulphuric acid, it well shaken then chloroform layer appeared red and acid layer shown greenish yellow fluorescence.
- Note: triterpenoid saponins are triterpenes which belong to the group of saponin.

Srividhya *et al.*,2015;..

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Tests for couramins:**
- Coumarins occur in plants in free form or as glycosides.
- Sweet-smelling plant substance called coumarin is found naturally in many plants, notably in high concentration in the tonka bean (*Dipteryx odorata*), vanilla grass (*Anthoxanthum odoratum*), sweet woodruff (*Galium odoratum*), mullein (*Verbascum* spp.), etc.
- For couramins analysis, 0.5g of moistened plant extract was taken in a test tube and covered with a filter paper moistened with 0.1N NaOH. The test tube was placed, for few minutes, in boiling water.
- Then the filter paper was removed and examined in UV light for yellow florescence to indicate the presence of couramins.

Jamil *et al.*,2012

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Test for steroids:**
- Plant sterols, or phytosterols, are naturally occurring plant molecules that are very similar to cholesterol.
- They are present in small quantities in many fruits, vegetables, vegetable oils, nuts.
- **Salkowski Test:** To 2 ml of ethanolic extract, 2 ml of chloroform and 2 ml of conc. H_2SO_4 was added.
- The solution was shaken well.
- As a result chloroform layer turned red and acid layer showed greenish yellow fluorescence.

Vindo *et al.*,2009

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Test for steroids(continued):**
- **Leibermann's Reaction:**
- 3 ml of ethanolic extract was mixed with 3 ml of acetic anhydride. The test solution was then heated and cooled. A few drops of conc. H_2SO_4 were added to the test solution.
- Appearance of blue colour shows the presence of sterols.

Vindo *et al.*,2009

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Tests for alkaloids(low molecular weight nitrogenous compounds):**
- 20% of plant species have been found to contain them.
- Examples: Nicotine, caffeine, morphine.
- The ethanolic extract was evaporated in a test tube. To the residue dilute HCl was added, shaken well and filtered. With the filtrate following tests were performed:
- **Hager's Test:** To the 2-3 ml of filtrate Hager's reagent was added.
- Yellow precipitate was formed showing the presence of alkaloids.
- **Mayer's Test:** To the 2-3 ml of filtrate Mayer's reagent was added.
- Formation of yellow precipitate showed the presence of alkaloids.

Vindo *et al.*,2009;..

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Tests for flavonoids:**
- Flavonoids are nearly ubiquitous in plants and are recognized as the pigments responsible for the colors of leaves, especially in autumn.
- They are rich in seeds(grape seed and flaxseed).
- **Shinoda Test:**
- To the ethanolic extract, added 5 ml of 95% ethanol and few drops of conc. HCl. To this solution 0.5 g of magnesium turnings were added.
- Observance of pink coloration indicated the presence of flavonoids.

Vindo *et al.*,2009;..

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Tests for flavonoids(continued):**
- **With Lead Acetate:** To the small quantity of ethanolic extract lead acetate solution was added. Formation of yellow precipitate showed the presence of flavonoid.
- **With Sodium Hydroxide:** On addition of an increasing amount of sodium hydroxide, the ethanolic extract showed yellow coloration, this decolorized after addition of acid.

Vindo *et al.*,2009

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Tests for phenolic compounds:**
- **$FeCl_3$ Solution Test for phenols:**
- On addition of 5% $FeCl_3$ solution to the ethanolic extract, deep blue black colour appeared.
- **Concentrated H_2SO_4 Test for Quinones:**
- The quinones are another group of phenolic compounds.
- To test the quinone phytochemical presence, in a test tube 1ml of extract and 1ml of concentrated sulphuric acid(H_2SO_4) was added.
- Formation of red colour shows the presence of quinones.

Vindo *et al.*,2009; Ganatra *et al.*,2012

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Tests for tannins:**
- Tannins are naturally occurring plant polyphenols.
- Their main characteristic is that they bind and precipitate proteins.
- Tannins are common in fruits (grapes, persimmon, blueberry, etc.), in tea, in chocolate, in legume forages (trefoil, etc.), in legume trees (*Acacia* spp., *Sesbania* spp., etc.), in grasses (sorghum, corn, etc.).
- To test the tannin phytochemical presence, in a test tube 1 ml of 5% ferric chloride added to solvent free extract.
- The presence of tannin is indicated by the formation of bluish black or greenish black precipitate.

Ganatra *et al.*,2012;..

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Tests for phlobatannins:**
- Phlobaphens can be formed under action of acids or heating of condensed tannins or of the fraction of tannins called phlobatannins.
- Phlobatannins were found in medicinal and aromatic plants.
- Deposition of a red precipitate when 0.25g of plant extract was boiled with 5mL of 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatinnins.

Jamil *et al.*,2012;..

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Test for proteins:**
- **Millon's test:** Test sample (3 ml) was mixed with 5 ml of millon's reagent. White precipitate is formed. On warming precipitate turn's brick red or the precipitate dissolves giving red colored solution.
- **Biuret test:** Test sample (3 ml) was mixed with 4% NaOH and few drops of 1% $CuSO_4$solution were added. Violet or pink color not appeared. To 3 ml of the extract few drops of 10% sodium chloride and 1% copper sulphate was added for the formation of violet or purple colour. On addition of alkali, it becomes dark violet.

Srividhya *et al.*,2015

Methods

Preliminary phytochemical screening

Phytochemicals present in the whole plant extract

- **Tests for amino acids:**
- **Ninhydrin test:** Test sample (3 ml) and 3 drops of 5% ninhydrin solution were heated in boiling water for 10 mins.
- Purple color appeared.

Srividhya *et al.*,2015

Methods

Thin-Layer Chromatography (TLC)analysis

Crude extracts of Thyme leaves

- Thyme leaves extracted with various solvents (acetone, chloroform, ethanol, ethyl acetate, hexane, methanol, methylene chloride, and petroleum ether) and constituents were analyzed quantitatively by TLC.
- Samples were diluted in ethanol (100 mg/mL) and spotted on a 250 µm 10 x 10 mm silica gel 60 F_{254} plates (EMD Chemicals).
- Next, the plates were developed in 8:4:1 hexane, dichloromethane, acetone system and allowed to air-dry in a fume hood.
- Plates were then analyzed under an ultraviolet light for separation of compounds based on polarity.

Nagy,2010

Methods

Thin-Layer Chromatography (TLC)analysis

Crude extracts of Thyme leaves

- Based on TLC results, it appears that the active component can be extracted in all the solvents tested, but the hexane extract contained the least number of inactive compounds.
- Hexane was also an easier solvent to work with based on its innate properties such as boiling point and hexane's ability to remove the least amount of undesirable compounds.

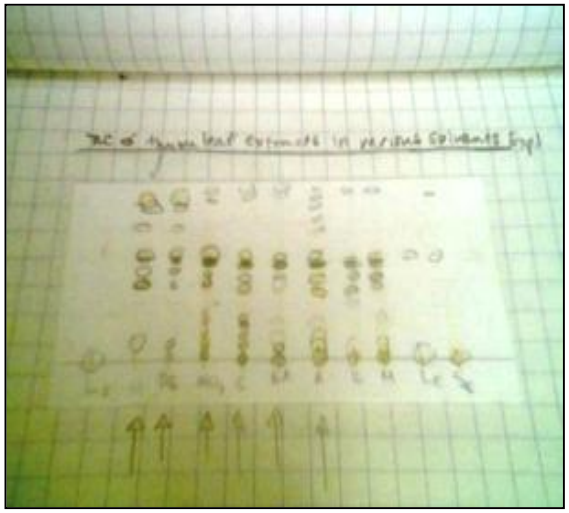
Nagy,2010

Methods

Thin-Layer Chromatography (TLC)analysis

Crude extracts of Thyme leaves

- TLC comparison of crude extracts of thyme leaves prepared with various solvents.
- Thyme leaves extracted in (by row): (1) ethanol, (2) hexane, (3) petroleum ether, (4) methylene chloride, (5) chloroform, (6) ethyl acetate, (7) acetone, (8) ethanol, (9) methanol, and (10) ethanol and (11) stems extracted in ethanol.



Nagy,2010

Methods

Thin-Layer Chromatography (TLC)analysis

Crude extracts of Thyme leaves

- This is the TLC plate of the ethanol and hexane crude extracts of thyme plus the four major constituents.
- Rows: (1) ethanol crude of thyme leaves, (2) thymol, (3) rosmarinic acid, (4) baicalein, (5) carvacrol, (6) internal control, and (7) hexane leaf extract.

Baicalein (98%), rosmarinic acid (97%), and liquid carvacrol (98%) were purchased from Sigma-Aldrich. Baicalein is a flavone compound that can be isolated from the methanolic extract of *Thymus vulgaris* leaves.

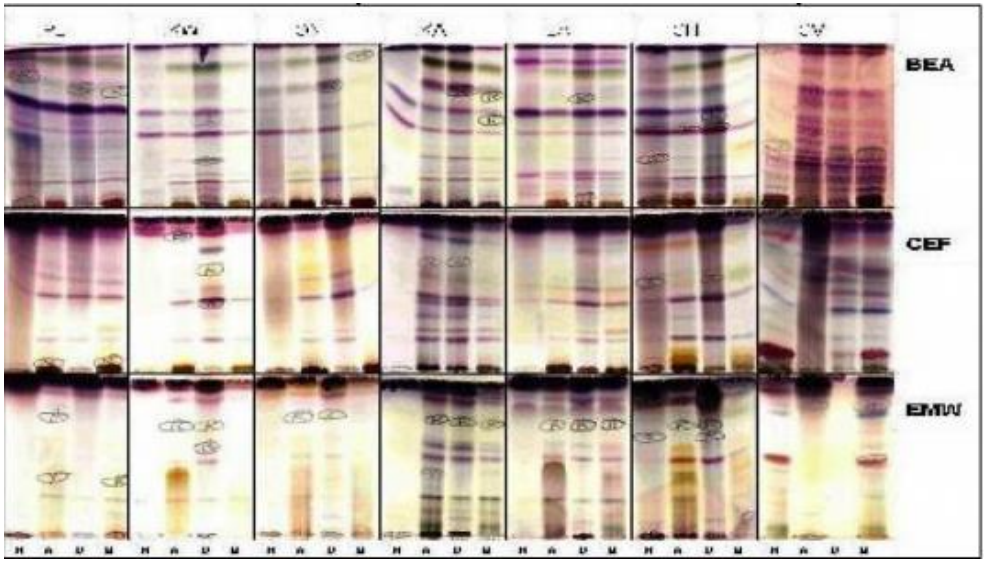
Nagy,2010

Methods

Thin-Layer Chromatography (TLC)analysis

Phytochemical analysis: detection with acidified vanillin

- Separated chemical compounds were detected using acidified vanillin (0.1 g vanillin: 28 ml methanol:1ml sulphuric acid) as a spray.
- After spraying, the chromatograms were heated at 110°C in an incubator to allow for optimal colour development.



Suleiman *et al.*,2010

Methods

Thin-Layer Chromatography (TLC)analysis

Calculation of RF value

- Calculation of Rf value is done with this formula:

$$Rf = \frac{\text{Distance traveled by solute from solvent front}}{\text{Distance traveled by solvent from solvent front}}$$

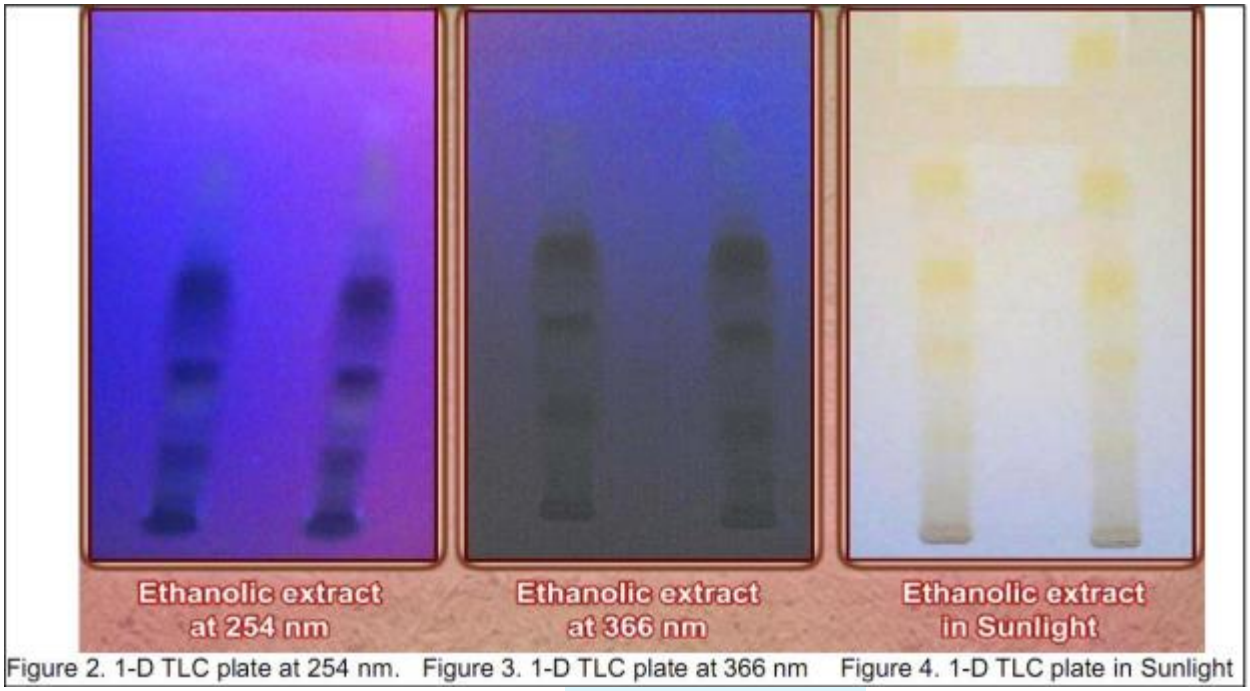


Figure 2. 1-D TLC plate at 254 nm. Figure 3. 1-D TLC plate at 366 nm Figure 4. 1-D TLC plate in Sunlight

Sharma,2012

Methods

Detection of antimicrobial compounds by bioautography of different extracts on TLC

Detection with *p*-iodonitrotetrazolium violet

- The acetone crude extract of the plant material was analyzed by silica gel TLC plates (ethyl acetatehexane = 1:1 v/v, as the mobile phase).
- A suspension of the gram positive bacteria, *Bacillus subtilis* (IFO 12113) and the gram negative bacteria, *Escherichia coli* (IFO 3301) in nutrient broth were sprayed on the developed TLC plates.

1. The TLC plates were incubated overnight at 25°C.
2. The TLC plates then sprayed with INT (*p*-iodonitrotetrazolium violet) solution (0.5 g/100 ml H_2O) and incubated to visualize the purple color.
3. Three white spots, representing the antibacterial constituents, two in the middle and one on the base line of the chromatogram were observed in case of *Bacillus subtilis*.

Jassbi *et al.*,2002

Methods

Antimicrobial/bacterial assays

Paper disk diffusion method

- After purification of antibacterial components by flash column chromatography, three compounds 1, 2 and 3 were subjected to antibacterial tests using different amounts.
- Compounds 1, 2, and 3 exhibited antibacterial activity against *Bacillus subtilis*, but were inactive against the gram negative bacteria at the presented amount in Table II (A).
- The dried papers were placed on agar seeded with *Bacillus subtilis* in a Petri dish.
- Pentachlorophenol(PCP) was used as the positive antibacterial control in the appropriate amounts shown.
- The antibacterial activity was determined by measuring the diameters of the clean inhibitory zone around each paper disk.

Jassbi *et al.*,2002

Methods

Antimicrobial/bacterial assays

TLC bioautography and Paper disk diffusion methods

- Table II (A and B) show the results of antibacterial activities of sclareol extracted from *Astragalus brachystachys* and its derivatives on TLC bioautography and disk diffusion tests (against *Bacillus subtilis*), respectively.
- Bioautography: An analytical technique in which organic compounds are separated by chromatography and identified by studying their effects on microorganisms.

Test Organism	1	2	3	PCP
Bacillus subtilis	100 / 30 a 50 / 25 20 / 14	100 / 20 50 / 15 20 / 13	100 / 11 50 / 8 20 / 8	2.3 / 19 1.6 / 17 1 / 15

B: by paper disk diffusion method.

Test Organism	1	2	3	PCP
Bacillus subtilis	200 / 18 b 160 / 11 120 / 10 80 / 9 40 / 8	200 / 0 160 / 0 120 / 0 80 / 0 40 / 0	200 / 0 160 / 0 120 / 0 80 / 0 40 / 0	20 / 32 16 / 31 12 / 27 8 / 23 4 / 0

Jassbi *et al.*,2002

Methods

Detection of antimicrobial compounds by bioautography of different extracts on TLC

Detection with *p*-iodonitrotetrazolium violet

- 10 µl (10 mg/ml) of each extract were loaded onto TLC plates in a narrow band and eluted using the three different mobile solvent systems (CEF, BEA and EMW).
- The developed plates were dried under a stream of fast moving air for 5 days to remove traces of solvent on the plates.
- Overnight cultures grown on Mueller Hinton broth were used and the densities of bacterial organism used for *E. faecalis*, *E. coli*, *P. aeruginosa* and *S. aureus* were approximately 2×10^{10}, 3×10^{11}, 5×10^{13} and 3×10^{12} cfu/ml, respectively.

ethyl acetate/methanol/water (40:5.4:5): [EMW](polar/neutral);
chloroform/ethyl acetate/formic acid (5:4:1): [CEF] (intermediate polarity/acidic);
benzene/ethanol/ammonia hydroxide (18:2:0.2): [BEA] (non-polar/basic)

Suleiman *et al.*,2010

Methods

Detection of antimicrobial compounds by bioautography of different extracts on TLC

Detection with *p*-iodonitrotetrazolium violet

- The prepared chromatograms were sprayed with bacterial suspension until wet.
- Thereafter, the plates were incubated overnight at 35°C and 100% relative humidity in the dark and then sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (INT) and further incubated overnight.
- White bands indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of tested organisms.

Suleiman *et al.*,2010

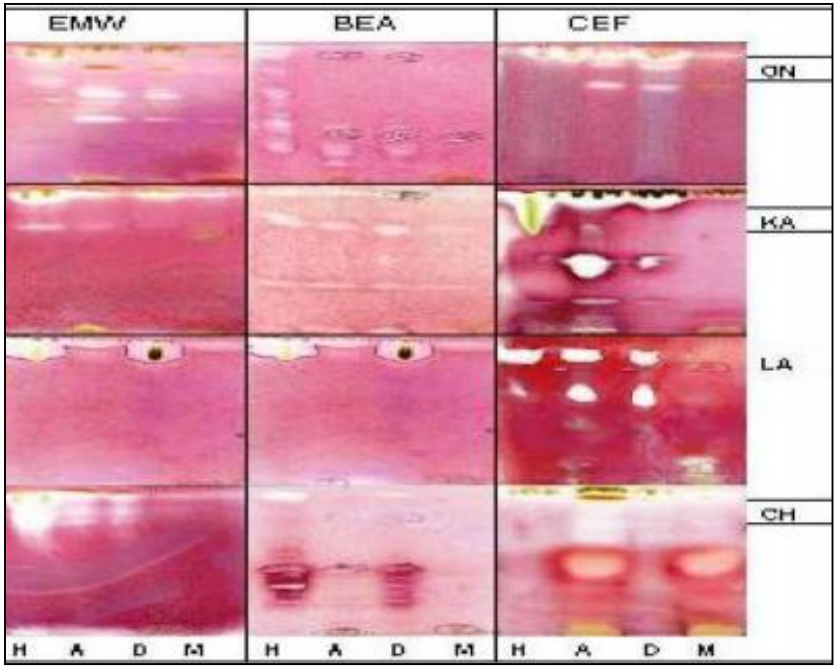
Methods

Detection of antimicrobial compounds by bioautography of different extracts on TLC

Detection with *p*-iodonitrotetrazolium violet

- Hexane (H), acetone (A), dichloromethane (D), and methanol (M) extracts of *Ochna natalitia* (ON), *Khaya anthotheca* (KA) *Loxostylis alata* (LA) and *Commiphora harveyi* (CH) separated on TLC plates using EMW, BEA and CEF, sprayed with bacterial organisms and 24 hrs later by INT.
- White areas indicate inhibition of bacterial growth by compounds of the plant extract after 60 mins of incubation at 37°C.

1. ON and KA were sprayed with *E. coli* while
2. LA and CH were sprayed with *S. aureus*.



Suleiman *et al.*,2010

Methods

Extraction, detection, purification and identification of quorum sensing molecules(AHLs or HSLs)

- Signal molecules should be extracted from bacterial culture supernatants.
- The difficulties in extraction of HSLs arise from the fact that there are many components present in the cell culture supernatants.
- The components are from the growth media and extracellular products produced by bacterial cells.
- To reduce the concentration of extracellular products, stationary cell growth phase is recommended for extraction.
- The two most commonly used methods to isolate HSLs are:

1. Liquid-liquid extraction (LLE), and
2. solid-phase extraction (SPE).

Wang *et al.*,2011

Methods

Detection of QS molecules from bacteria

- Extracts for analytical TLC were prepared from 5-ml cultures, while those used to process samples for analysis by MS(mass spectrometry) were prepared from 500-ml cultures.
- Bacteria were removed by centrifugation, the supernatants were extracted twice with equal volumes of ethyl acetate, and the combined extracts were dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness.
- Residues from 5-ml cultures were dissolved in 50-100 µl of HPLC-grade ethyl acetate.
- Residues from 500-ml cultures were dissolved in the minimum volume of HPLC-grade ethyl acetate, usually 20-50 µl.

Shaw *et al.*,1997

Methods

Detection of QS molecules from bacteria

Isolation of autoinducer (C6 AHL) from CV31532

- *Chromobacterium violaceum* CV026 is a mini Tn5 mutant indicator strain derived from wild type *C. violaceum* CV31532 strain.
- C6 AHL was obtained by growing *C. violaceum* CV31532 strain, an over producing stain in Luria broth (4L) on shaking incubator at 28°C for 18 h.
- The filtrate obtained by centrifugation(12000 rpm, 10 min) was sterilized by Millipore (0.22 µm).
- The filtrate obtained was extracted with acidified acetyl acetate(0.1% v/v acetic acid) in ratio 7:3 (supernatant: acidified acetyl acetate v/v) and finally concentrated and dried by rotary evaporator at 40°C and reconstituted in acetonitrile and stored at 4°C for future use in bioassay using CV026.
- The amount of partially purified AHL needed for production of violacein by CV026 was first standardized by plate agar.

Zahin *et al.*,2010

Methods

Detection of QS molecules from bacteria

Extraction (Isolation) of autoinducer(AHL) from *Bradyrhizobium*

- Extraction of AHLs was performed as described by Shaw *et al.*,1997.
- Peanut-nodulating Bradyrhizobia were grown to the early stationary phase, and cells were removed from 25 mL growth medium by centrifugation at 12,000 g for 15 min at 4°C.
- AHLs were then extracted from culture supernatants with three equal volumes of ethyl acetate (3:3 supernatant: acetyl acetate v/v), and the extracts were dried and resuspended in 500 µL ethyl actetate.

G force depends on rpm and the radius. You can use the following equation for the conversion:
g Force (RCF) = $(rpm)^2 \times 1.118 \times 10^{-5} \times r$ (where r is the rotational radius in centimeter).
More details available at: G Force RPM Calculator -- EndMemo

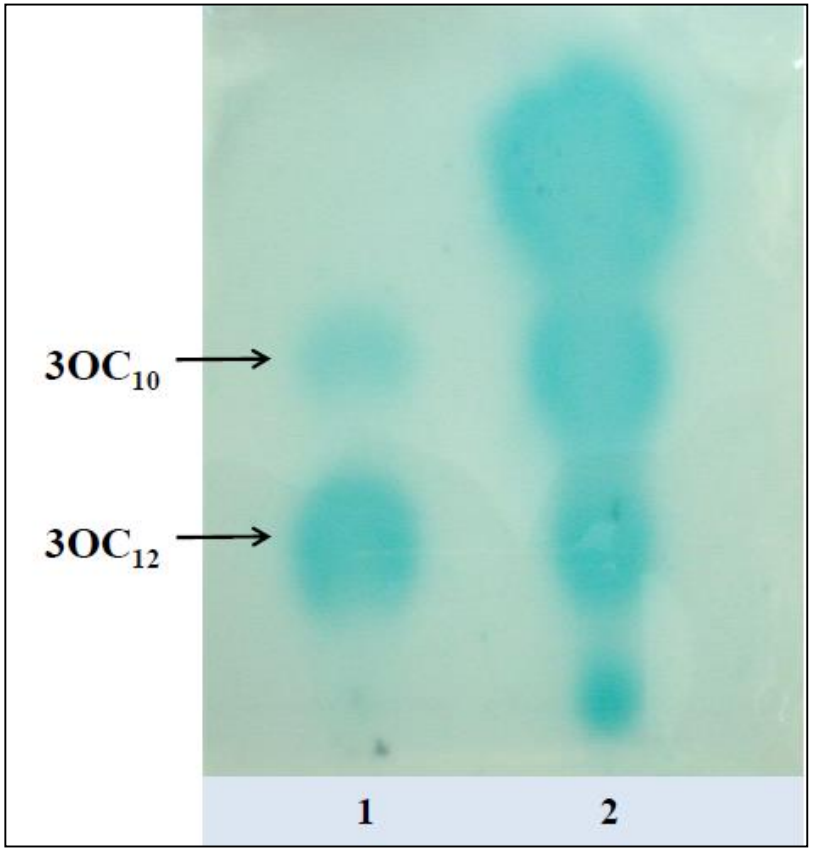
Nievas *et al.*,2012

Methods

Detection of QS molecules from bacteria

Isolation of autoinducer(AHL) from *Bradyrhizobium*

- Detection of AHLs with long acyl chain by thin layer chromatography.
- The TLC plate was overlaid with the biosensor *A. tumefaciens* NTL4 (pZLR4).
- Lane 1, long chain 3-oxo-standards(3OC10, 3OC12).
- Lane 2, 10 µL ethyl acetate extract from supernatant of liquid culture of bacterial strain 62B.



Nievas *et al.*,2012

Methods

Detection of QS molecules from bacteria

Extraction of autoinducer (C8 AHL) from antiQS-bacteria

- Bacterial cells were removed by centrifugation at 12000 rpm for 5 min. The culture supernatant was extracted twice with equal volumes of ethyl acetate. The organic phase was taken to dryness under an evaporator.
- Residues were redissolved in 50 ml volumes of ethyl acetate and store at -20°C. Component in the ethyl acetate extracts were separated by chromatography on C-reversed phase plate with a solvent system of methanol-water (60:40, vol/vol) at room temperature.
- After development, the solvent was evaporated, and the dried plates were overlaid with a culture of the biosensors bacteria such as *Chromobacterium violaceum* (Shaw *et al.*, 1997 and McClean *et al.*,1997).

Mahmoudi *et al.*,2011

Methods

Detection of QS molecules from bacteria

Isolation of autoinducer (C8 AHL) from *Acinetobacter*

- Bacterial cultures were centrifuged at 12,000 *g* for 10 min and the resulting supernatants were filtered through 0.22 µm PVDF membrane filters (Millipore).
- Supernatants were then extracted twice with 30 ml of ethyl acetate; subsequently the extracts were pooled, dried on anhydrous Na_2SO_4 and the solvent evaporated in a rotary evaporator at 40°C.
- The extracts were then resuspended in 100 µl methanol and kept frozen at -20°C.

1. Fresh culture medium (30 ml) was similarly extracted and used as a control.
2. A solution of 2 mg/ml octanoyl-L-homoserine lactone (C8-HSL, Fluka) in methanol was used as a reference compound.

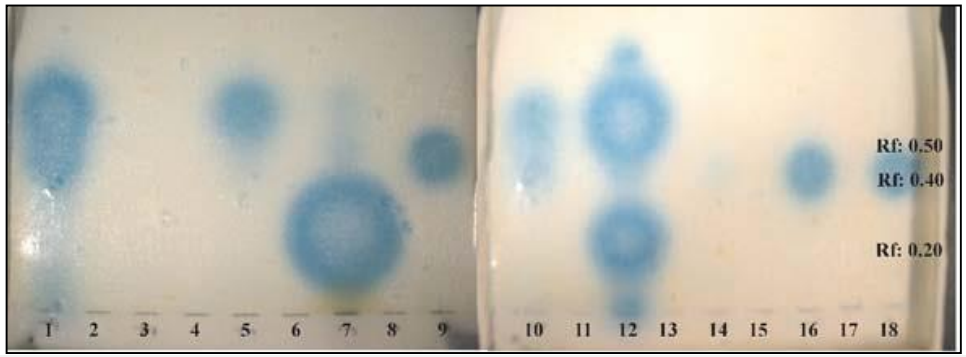
González *et al.*,2009

Methods

Thin-Layer Chromatography (TLC)analysis

Isolation of autoinducer (C8 AHL) from *Acinetobacter*

- 10 µl of each of the resuspended extracts, 2 µl of C8-HSL reference solution and 10 µl of the control extracts were run on normal phase silica Gel GF 254 plates and developed with a mixture of chloroform: methanol (95:5, v/v).
- Plates were dried at room temperature in a laminar flow cabinet.



Lanes 9 and 18 as reference compound (C8-HSL), 8 and 17 as control extract.

After drying the plate, 1 ml of a suspension of the *Agrobacterium* reporter strain and 200 µl of 20 mg/ml X-gal solution in dimethyl formamide were added to 100 ml of M9 medium with 0.9% agar at 40°C and poured on the TLC plate which was incubated at 28°C for 24 h.

González *et al.*,2009

Methods

Detection of QS molecules from bacteria

Isolation of autoinducer (C8 AHL) from *Agrobacterium vitis*

- Signal extraction and detection bacterial culture supernatants resuspended from PDA plates were extracted with acidified ethyl acetate (aEtOAc) (1 mL of glacial acetic acid per 200 mL of ethyl acetate) for 30 min with shaking (150 r.p.m.) and centrifuged to separate the aqueous and ethyl acetate phases.
- The ethyl acetate phase was recovered and dried in a Savant Speed Vac.
- Twentyfold (fold indicates concentration. E.g. double and triple strength (up to 20-fold) concentrated extracts were used in AHL assays.
- AHLs were determined using five different reporter bacteria in which a detectable phenotype depends upon the LuxR-homolog's ability to sense exogenous AHLs.
- Extractions were screened using the bioreporter *A. tumefaciens* NTL4 (pZLR4) in well-diffusion assays before performing thin-layer chromatography (TLC)-overlay detection assays (Shaw *et al.*, 1997 and Farrand *et al.*, 2002).

Lowe *et al.*,2009

Methods

Detection of QS molecules from bacteria

Isolation of autoinducer (C8 AHL) from *Pantoea*

- Isolate M009 was cultured in LB broth buffered to pH 5.5 with 50 mM of 3-(*N*-morpholino) propanesulfonic acid (MOPS) in an incubator shaker (200 rpm; 28°C; 18 h).
- The cultured supernatant was extracted twice with equal volume of acidified (0.1% v/v glacial acetic acid) ethyl acetate as described previously.
- The organic solvent was dried in fume hood and the dried extracts were resuspended in 1 mL of acidified ethyl acetate and desiccated completely.
- Finally, 200 µL of acetonitrile (HPLC grade) was added and vortexed to dissolve the dried extracts.
- The extract was then centrifuged at 12,000 rpm for 5 min to remove any insoluble residue. The dissolved sample with 75 µL aliquot was withdrawn from the upper layer and inserted in sample vials for mass spectrometry Analysis.

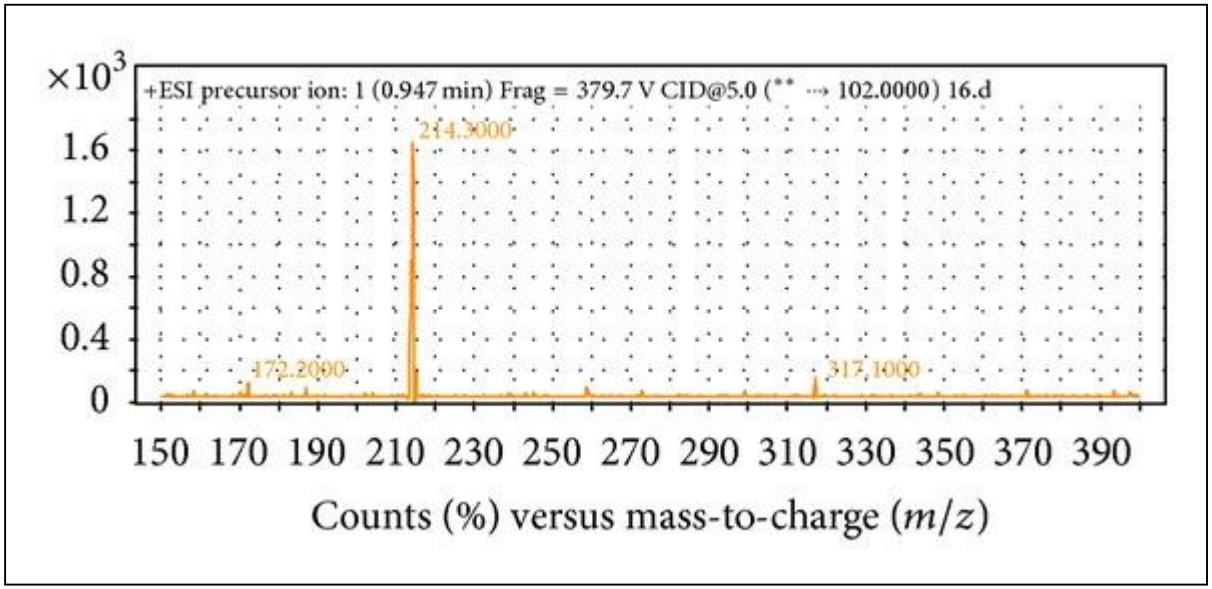
Tan *et al.*,2014

Methods

Detection of QS molecules from bacteria

Isolation of autoinducer (C8 AHL) from *Pantoea*

- Mass spectrometry analysis of AHL extracts from *Pantoea* sp. strain M009.
- Mass spectra of 3-oxo-C6 HSL (*m/z* 214.3000) was detected.



Tan *et al.*,2014

Multilingual bacteria

Presence of multiple QS signals(AHLs) in culture supernatants

- The presence of multiple QS signals in culture supernatants of gram-negative bacteria is not a rare finding.

1. For instance in *Pseudomonas aeruginosa*, in which quorum sensing systems have been extensively characterized, two main AHSLs are produced (C4-HSL and 3oxo-C12-HSL), while the same signaling systems can synthesize other AHSLs in smaller amounts (3-oxo-C6-HSL and C6-HSL) as well.
2. In *Pseudomonas aureofaciens*, the main QS signal was C6-HSL, which is also a regulator of the synthesis of the antibiotic phenazine.
3. In *Pseudomonas putida*, 3-oxo-C10-HSL and 3-oxo- C12-HSL are the main QS signals, and 3-oxo-C8-HSL and 3-oxo-C6-HSL are secreted in minor amounts.

González *et al.*,2009

Methods

Detection of QS molecules(AI-1 and AI-2)

Using the *Vibrio harveyi* bioluminescence assay

- The cell-free bacterial supernatant was prepared at different time points by centrifugation of the sample at 10.000 × g for 10 minutes.
- The supernatant was immediately frozen at -80°C until being subjected to AI measurements:
- A 60 µL aliquot of each sample was added to 600 µl of the sensor bacterium *V. harveyi* BB170 (AI-2) or *V. harveyi* BB886 (AI-1) (being diluted 1:5.000 after being grown in "AB" medium) and was incubated at 30°C.
- Every hour the bioluminescence was measured using a Wallac Luminometer.
- All measurements were reported at the three hour incubation period, when the difference between negative controls and positive controls reached maximal levels.

Jacobi *et al.*,2012

Methods

Thin-Layer Chromatography (TLC)analysis

AHL profiles of the rhizosphere isolates

- TLC in combination with AHL biosensors provides a simple and rapid technique for characterizing and quantifying the AHL species produced by a given organism.
- Dichloromethane extracts of spent culture supernatants of *P. putida* IsoF, *P. putida* Z2D, *R. aquatilis* TAA, and *R. aquatilis* T13 were analyzed and both *P. putida* strains, Z2D and IsoF, exhibited very similar AHL patterns.
- Using the bioluminescent monitor strain *E. coli* MT102(pSB403), four different AHL species were detected(bioluminescence was measured using a Berthold LB96V luminometer).
- Based on their mobilities (*Rf* values) and by including appropriate reference compounds, these molecules were tentatively identified as 3-oxo-C12-HSL, 3-oxo-C10-HSL, 3-oxo-C8-HSL, and 3-oxo-C6-HSL.

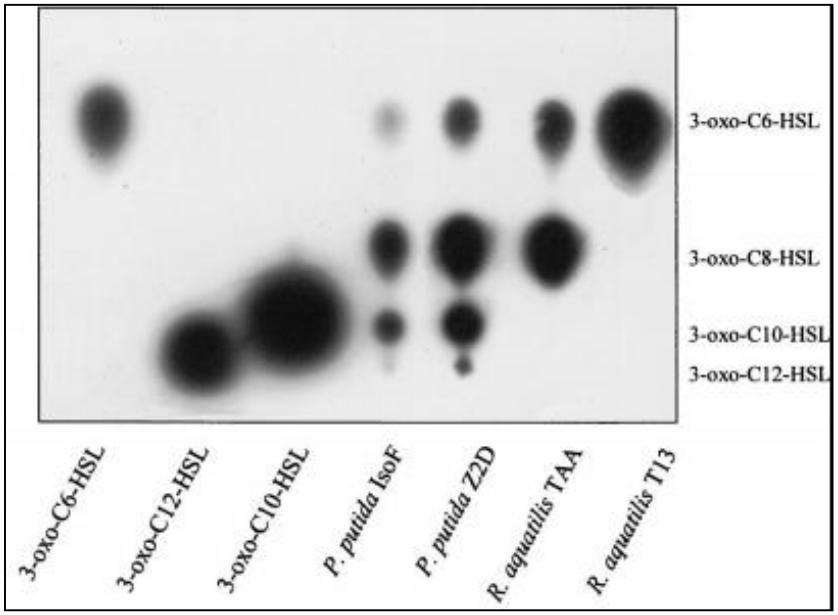
Steidle *et al.*,2001;..

Methods

Thin-Layer Chromatography (TLC)analysis

Isolation of autoinducer (C8 AHL) from *Acinetobacter*

- TLC analysis of AHLs produced by bacterial strains isolated from the tomato rhizosphere.
- AHLs extracted from cell-free culture supernatants of *P. putida* IsoF, *P. putida* Z2D, *Rahnella aquatilis* TAA, and *R. aquatilis* T13 were separated by TLC, and spots were detected with the aid of the AHL biosensor *E. coli* MT102 (pSB403) which best respond to C6-3-oxo-AHL.
- Synthetic AHLs were included as reference compounds, as indicated.



Steidle *et al.*,2001

Detection of QS molecules from bacteria

Isolation of autoinducers (C4 and C6 AHLs) from *Aeromonas Caviae* Strain YL12

- *Aeromonas caviae* YL12 produced two short chain *N*-acyl homoserine lactones(AHLs), namely C4-HSL and C6, and the production was observed to be cell density-dependent.
- Using the thin layer chromatography (TLC) bioassay,

1. both AHLs were found to activate *C. violaceum* CV026,
2. whereas only C6-HSL was revealed to induce bioluminescence expression of *E. coli* [pSB401].

Lim *et al.*,2014

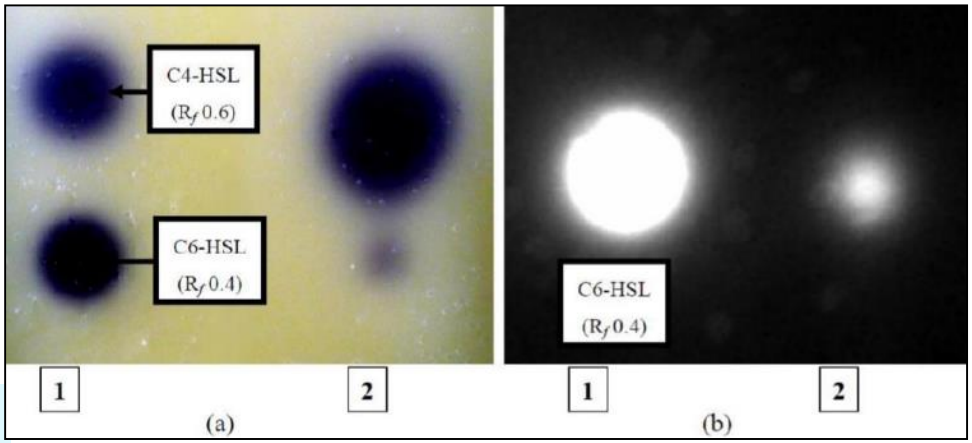
Detection of QS molecules from bacteria

Isolation of autoinducers (C4 and C6 AHLs) from *Aeromonas Caviae* Strain YL12

TLC bioassay of spent supernatants extracts of YL12

a) TLC/CV026 bioassay: shows that YL12 extracts (lane 2) were chromatographed into two distinct spots with R*f* value of 0.4 and 0.6, which corresponds to the R*f* values of the synthetic standards (lane 1) used, namely C4-HSL (R*f* value of 0.6) and C6-HSL(R*f* value of 0.4).

b) TLC/*E. coli* [pSB401] bioassay: showed only one bioluminescent spot in both lane 2 (YL12 extracts) and lane 1 (synthetic standard) with R*f* value of 0.4 following incubation at 37°C.



Lim *et al.*,2014

Methods

Quantification of Violacein

Violacein extraction

- Purple pigment violacein was produced when *C. violaceum* CV026 was exposed to the cognate autoinducer molecules C6-AHL and C4-AHL.
- Violacein extraction was carried out according as previously described by Blosser and Gray,2000.
- Briefly, bacterial cells were lysed by 10% sodium dodecyl sulfate and incubating at room temperature for 5 minutes.
- Water-saturated butanol was added, vortexed, and centrifuged at 13000 rpm for 5 minutes.
- The upper(butanol) phase containing the violacein was collected.
- The extracted violacein was quantified using a spectrophotometer (optical density at 585 nm wavelength).

Bosgelmez *et al.*,2007

Methods

Violacein extraction

Quantifying violacein production

- Violacein extraction was carried out according as previously described by Blosser and Gray,2000.
- The LB tubes grown *C. violaceum* CV026 were vortexed to resuspend cells and biofilms; and 300 µl of bacterial cells suspension were placed in 1.5 ml Eppendorf tubes.
- The cells were lysed with 300 µl of 10% sodium dodecyl sulfate, vortexed for 2 minutes, and then incubated at room temperature for 5 minutes.
- Violacein was extracted quantitatively adding 800 µl of a mixture of butanol/water 1:1, stirred for 5 seconds and then centrifuged at 13000 rpm for 5 minutes.
- Once centrifuged, the violacein, which was present at the upper layer, was carefully removed and its absorbance measured at 585 nm.

Olivero *et al.*,2011

Methods

Violacein extraction

Quantifying violacein production

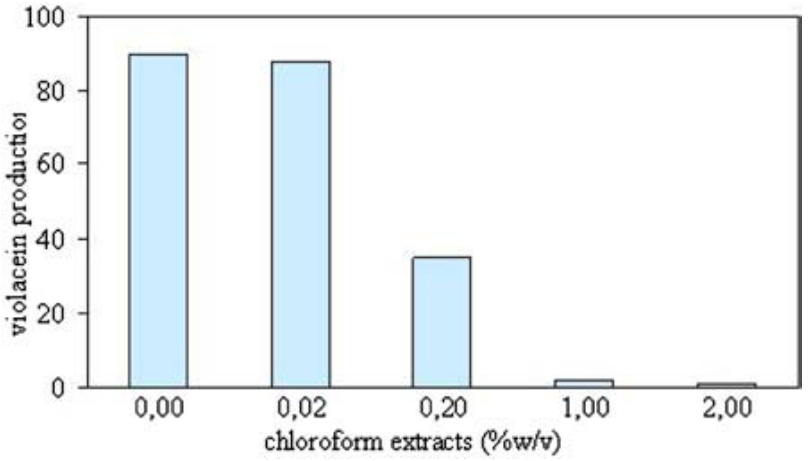
- The production of violacein was quantified as follows:
- Tubes containing overnight culture and treatment were vortex for 30 seconds to re-suspend any pellicle of adherent cells after 16–18 hours incubation.
- Bacterial culture (1 mL) of each tube was centrifuged at 13,000 rpm for 10 minutes to precipitate the insoluble violacein.
- The cultures supernatant was discarded and 1 mL of 100% DMSO was added to the pellet.
- The solution was then vortex vigorously for 30 seconds to ensure that the violacein has completely solubilized and centrifuged at 13,000 rpm for 10 minutes to remove the cells.
- Then, 200 µL of the violacein-containing supernatants were then added into 96-well flat-bottomed microplate (SPL Life Sciences, Pocheon-Si, Korea) in triplicate.
- The absorbance was read using the Tecan Infinite M200 luminometer at a wavelength of 585 nm.

Norizan *et al.*,2013

Methods

Effect of plant extracts on violacein production

- Violacein production in *C. violaceum* CV026 was induced by C6-HSL.
- Plant extracts obtained from chloroform were added to *C. violaceum* CV026 cultures at conc. of 0%, 0.02%, 0.2%, 1%, and 2% (%w/v).
- C6-HSL–inducible violacein production was inhibited by a chloroform extract of *S. sandrasica* (Compositae) family in proportion to the amount of extract added.



Inhibitory activity of chloroform extract on violacein production was also measured spectrophotometrically.

Bosgelmez *et al.*,2007

Methods

Violacein extraction

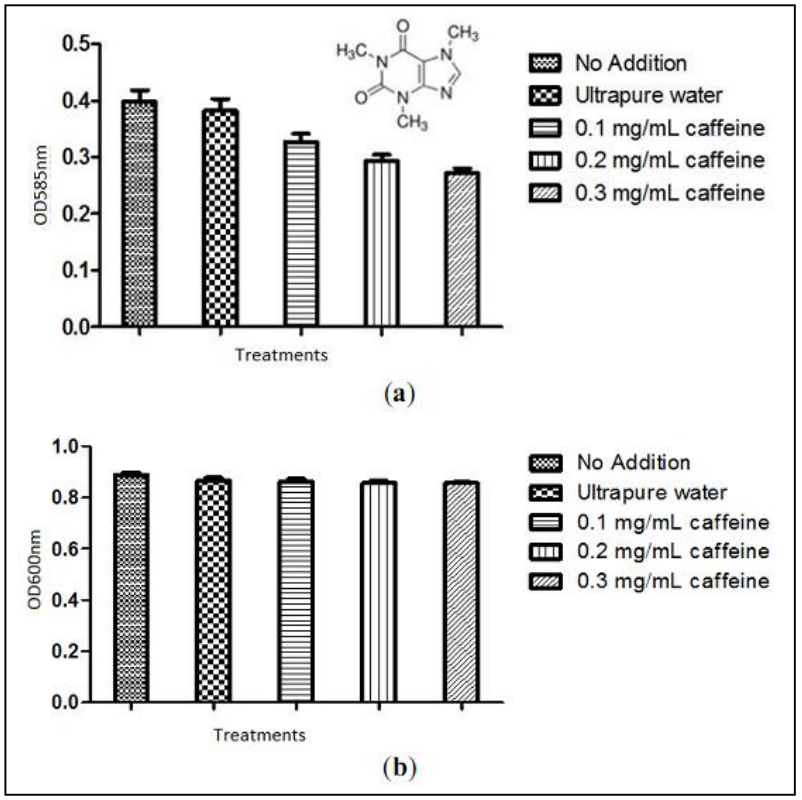
Quantifying violacein production

- Caffeine as a bioactive compound a natural pesticide inhibits CV026 violacein production by anti-QS.
- The violacein production was measured spectrophotometrically and quantified by reading the OD values of the solution at:

a) 585nm, and

b) Bacterial growth at 600 nm.

- The statistical significant of each test (n=3) was evaluated by conducting one-way ANOVA test and a P value of $P < 0.05$ being significant.
- Inset: Structure of caffeine.



Bioactive compounds in plants can be defined as secondary plant metabolites eliciting pharmacological or toxicological effects on a living organism.

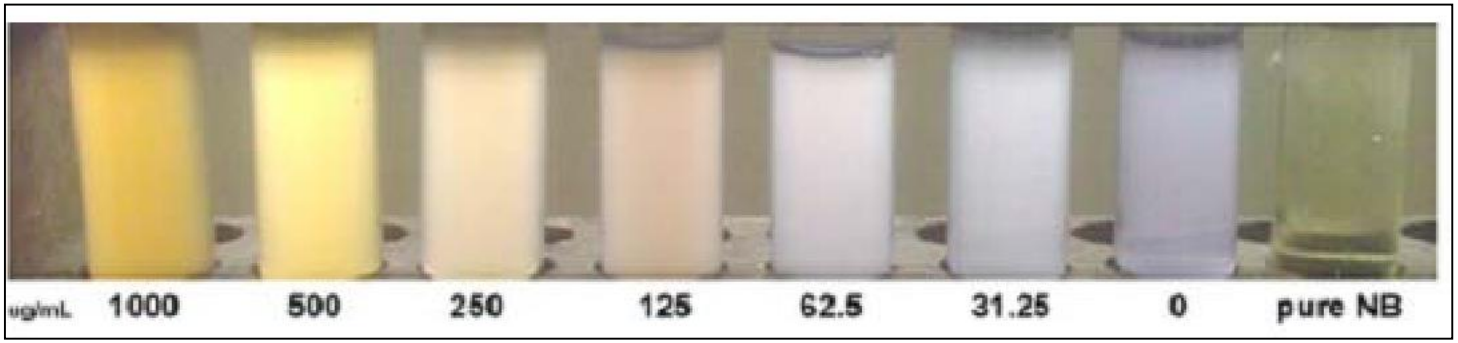
Norizan *et al.*,2013;..

Methods

Effect of (EGCG) from green tea on violacein production

Limits of violacein detection

- Two-fold serial dilution of the anti-QS substance Epigallocatechin gallate (EGCG) from green tea(*Camellia sinensis*) was prepared in 8-well lanes of a 96-well microtiter plate (1000, 500, 250, 125, 62.5, 31.25, 15.625 and 0 µg/mL) at 150 µl each using LB broth as diluent.
- Photograph showing the increasing production of violacein by *C. violaceum* at decreasing concentrations of EGCG.

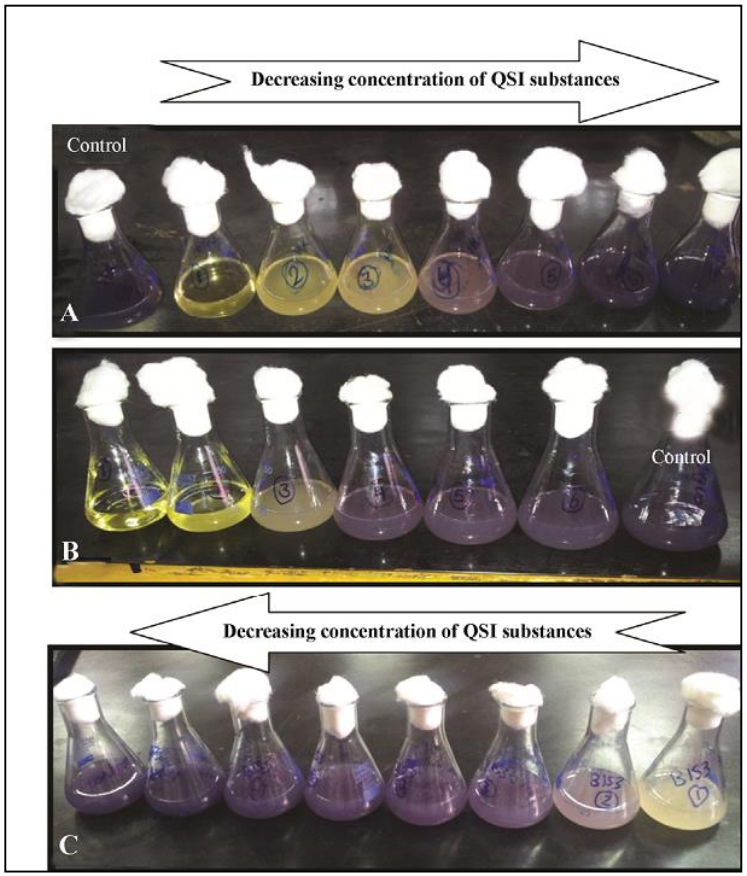


Taganna and Rivera,2008

Methods

Quantification of violacein by flask method

A. hexane QSI substances of isolate S 664 extract,
B. butanolic QSI substances of isolate S 130 extract, and
C. the QSI substances of isolate S 153 butanolic extract.

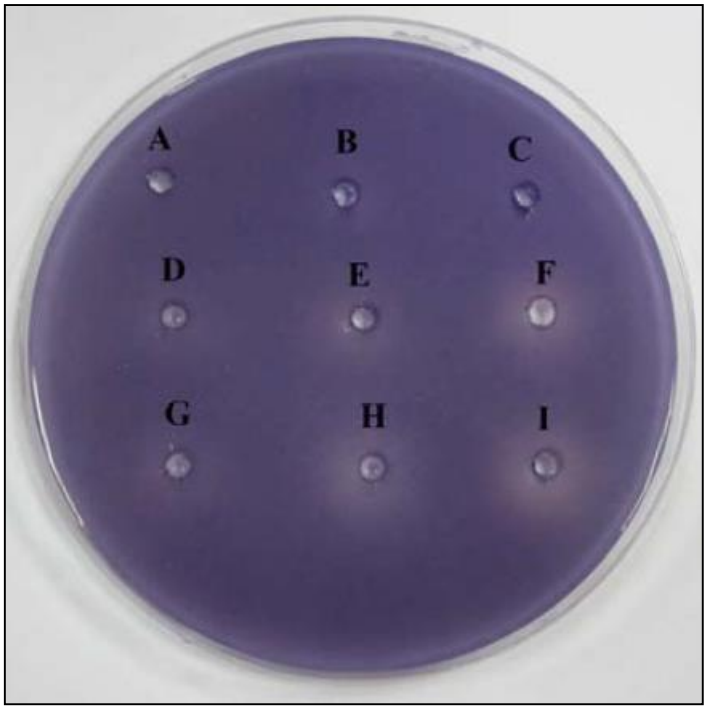


Abudoleh and Mahasneh,2017

Methods

Anti-QS property of plant extracts (caffeine and (+)-catechin) were not due to the degradation of the C6-HSL

- Figure showed that caffeine and (+)-catechin at the tested concentrations did not degrade C6-HSL.
- Disc (A) C6-HSL treated with ultrapure water (negative control);
- (B) C6- HSL treated with 20% DMSO (negative control);
- (C) PBS;
- (D-I) C6-HSL treated with different conc. of caffeine and (+)-catechin.
- The result showed that both caffeine and (+)-catechin have no effect on C6-HSL.



Halo zone formation on the purple background suggest that caffeine exhibited anti-QS property.

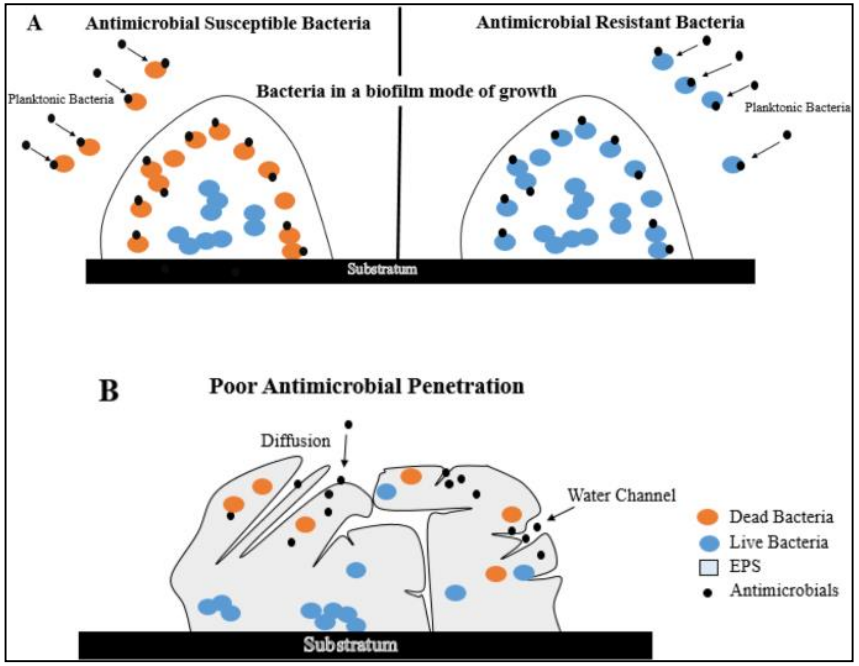
Norizan *et al.*,2013

QS/Biofilm control
Microbial aggregation

- A biofilm is a matrix-enclosed microbial aggregation that adheres to a biological or non-biological surface.
- On plant surfaces it is common to find assemblages of microorganisms adherent to each other and/or to a surface and embedded in a matrix of exopolymers.
- These matrices are referred to as biofilms.
- The QS signal molecule has been shown to mediate biofilm formation to protect against antibiotics.

Biofilm control
Microbial aggregation

- An alternative therapeutic techniques including modulating microbial metabolism, matrix degrading enzyme, photodynamic therapy, natural compounds quorum sensing and nanotechnology which are being used to disrupt extra polymeric substances (EPS) matrix of desired bacterial biofilms.



Khan *et al.*,2021

Biofilm control

A list of common biofilm forming bacterial species

- *Burkholderia cepacia*
- *Escherichia coli*
- *Pseudomonas aeruginosa*
- *Staphylococcus aureus*
- *Stereptococcus epidermidis*
- *Enterobacter cloacae*
- *Klebsiella pneumoniae*

Biofilm formation by plant bacterial pathogens

- *Xanthomonas campestris* pv.*campestris*
- *Pseudomonas syringae* pv.*syringae*
- *Dickeya chrysanthemi*
- *Pantoea stewartii* subsp.*stewartii*
- *Ralstonia solanacearum*
- *Clavibacter michiganensis* subsp.*sepedonicus*
- *Xylella fastidiosa*

Biofilm management
Damage estimation

- It has been estimated that billions of dollars are spent every year worldwide to deal with damage to equipment, contaminations of products, energy losses, and infections in human beings resulted from microbial biofilms.
- Microorganisms compete, cooperate, and communicate with each other in multi-species biofilms.
- Understanding the mechanisms of multi-species biofilm formation will facilitate the development of methods for combating bacterial biofilms in clinical, environmental, industrial, and agricultural areas.

Yang *et al.*,2011

Biofilm

- Biofilms are composed of an exopolymeric matrix and multiple layers of microbial cells leading to the creation of physical barriers and the establishment of chemical gradients.
- They generally contain multiple species of microorganisms, fostering metabolic and genetic exchange.
- Many biofilm cells are attached to a surface (biotic or abiotic, including the surface of other microorganisms or debris in the same biofilm).
- It is also known that biofilms can harbor strains in a VBNC (Viable but not culturable) state.

Strain variation is common in bacteria. Some strains and not all strains of a particular species are able to enter the VBNC state within a biofilm. E.g. *Campylobacter jjejuni* can become VBNC in response to various stressors, such as starvation, low temperature, and low pH.

Biofilm

Thickness and composition of biofilm matrices

- Biofilms can vary in thickness from a mono cell layer to 3 inch/7 cm thick layer but mostly on average are 100 micron or micrometer (=0.01 cm) thick.
- Chemical analysis shows that the EPS matrix is mostly comprised of neutral and acidic sugars, e.g. glucose, mannose, galactose, rhamnose, ribose, fucose, uronic and gluconic acids, etc.

Biofilm

Range of composition of biofilm matrices

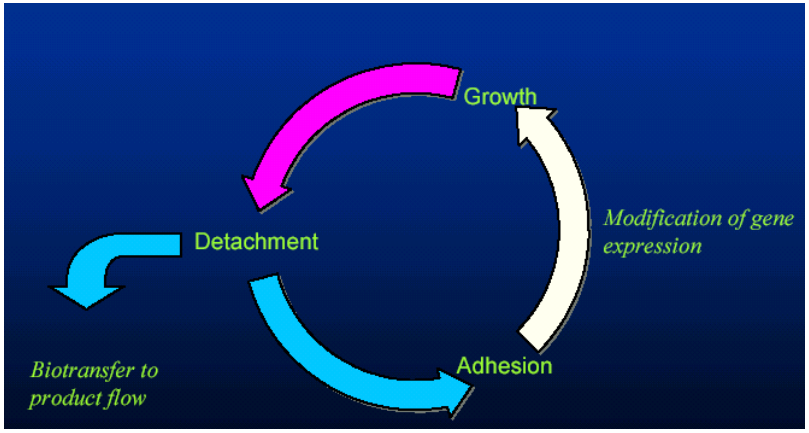
Component	% of matrix
Water	Up to 97%
Microbial cells	2-5% (Many species)
Polysaccharides (homo-and heteropolysaccharides)	1-2% (Neutral and polyanionic)
Proteins (extracellular and resulting from lysis)	<1-2% (Many, including enzymes)
DNA and RNA	<1-2% (From lysed cells)
Ions	? (Bound and free)

Sutherland,2001

Biofilm life cycle

- The biofilm life cycle in three steps:

1. attachment,
2. growth of colonies (micro-colony formation and
3. formation of three dimensional structures) and detachment in clumps.



Biofilm

Biofilm population versus solitary populations

- Numerous plant-pathogenic bacteria multiply or survive on aerial parts of plants without causing any visible symptoms.
- This asymptomatic phase allows bacterial populations to attain sizes permitting, in favorable environments, disease development.
- Sizes of these epiphytic populations are predictive of the amount of disease in some cases, and in other cases a threshold of population sizes is necessary to produce symptoms (Disease forecasting).
- It was concluded bacterial cell aggregation in biofilms on leaf surfaces provides protection to the bacterial cells against hydric stress (desiccation).

Biofilm

Biofilm population versus solitary populations

- Biofilm population sizes are always lower than solitary(existing alone) population sizes.
- Differences between biofilm population sizes and solitary(existing alone) population sizes can reach more than 10^2 CFU/g of fresh weight.
- On the other hand, biofilm population sizes remained stable throughout the growing season (around 10^5 CFU/g of fresh weight) while solitary population sizes were more abundant and varied with climate.
- e.g. "*Xanthomonas fuscans* subsp. *fuscans*"

Jacques *et al.*,2004

Biofilm

Biofilm population versus solitary populations

- It has been also confirmed solitary population sizes were more abundant and varied with climate.
- We demonstrated that biofilms offered more protection to the bacterial populations than did the solitary state; multiplication of the solitary fraction of the population was significantly ($P < 0.05$) altered after stress application, while biofilm population sizes were not affected.
- Only suppression of the hydric stress (desiccation) allowed solitary bacterial populations to increase again.

Jacques *et al.*,2004

Biofilm

Phyllosphere colonization

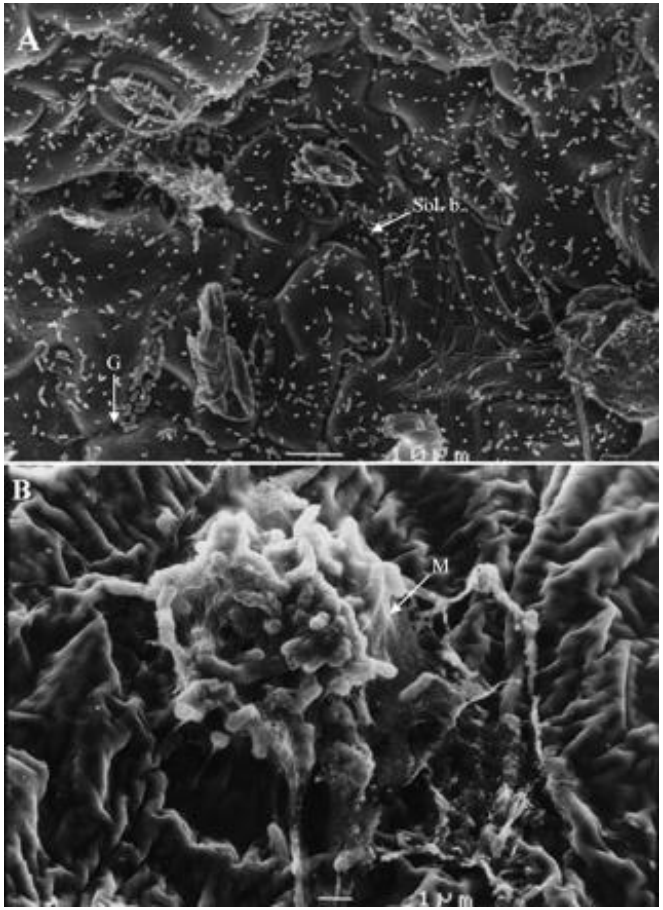
- Scanning electron microscopic micrographs of field-grown bean leaf surfaces colonized by seed-borne *X. axonopodis* pv. *phaseoli*.

(A) Leaf surface showing mostly solitary bacterial populations (Sol. b.).

- Note the accumulation of bacterial cells in grooves (G) between epidermal cells.

(B) Focus on a bacterial biofilm.

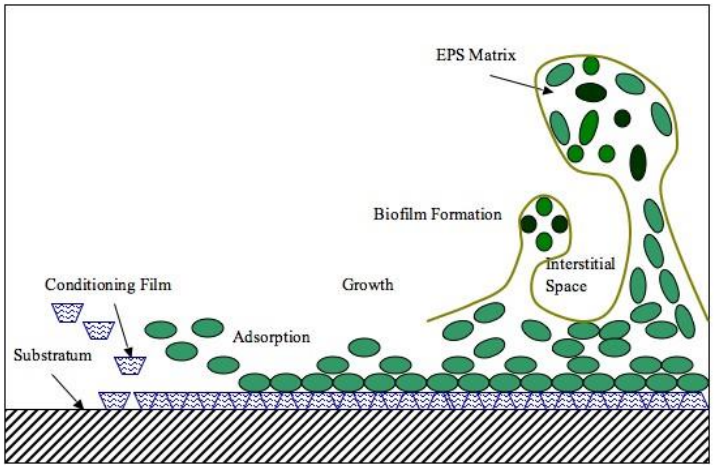
- Note the matrix (M) embedding bacterial cells constituting a typical biofilm.
- Bar, 1 µm.

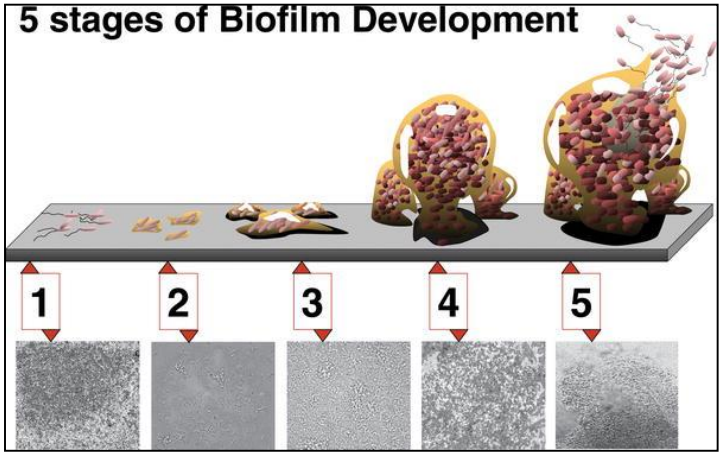


Steps in biofilm development

A glance of a developing *P. aeruginosa* biofilm

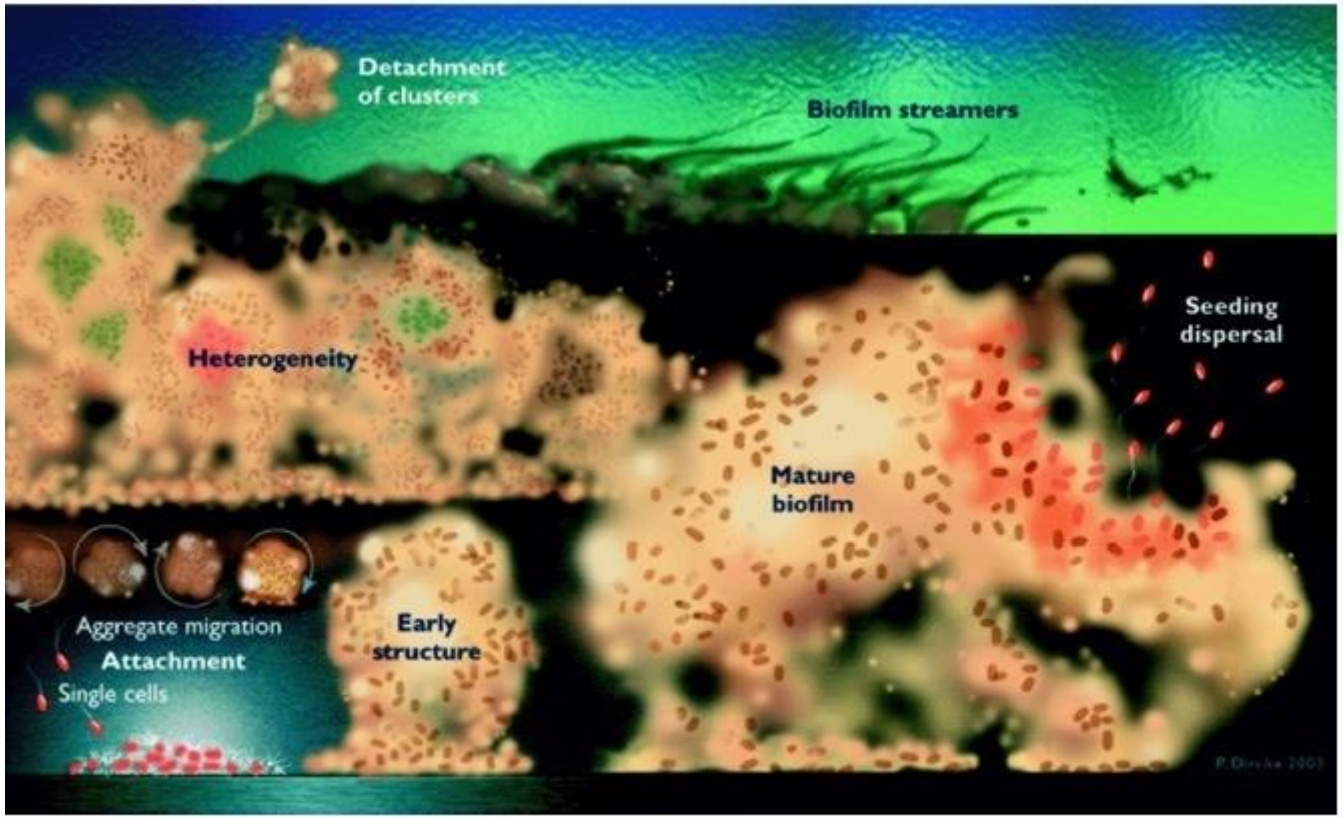
- Stage 1, initial attachment;
- Stage 2, irreversible attachment;
- Stage 3, maturation I;
- Stage 4, maturation II;
- Stage 5, The mature, fully functioning biofilm ready for dispersion.
- All photomicrographs are shown to same scale.





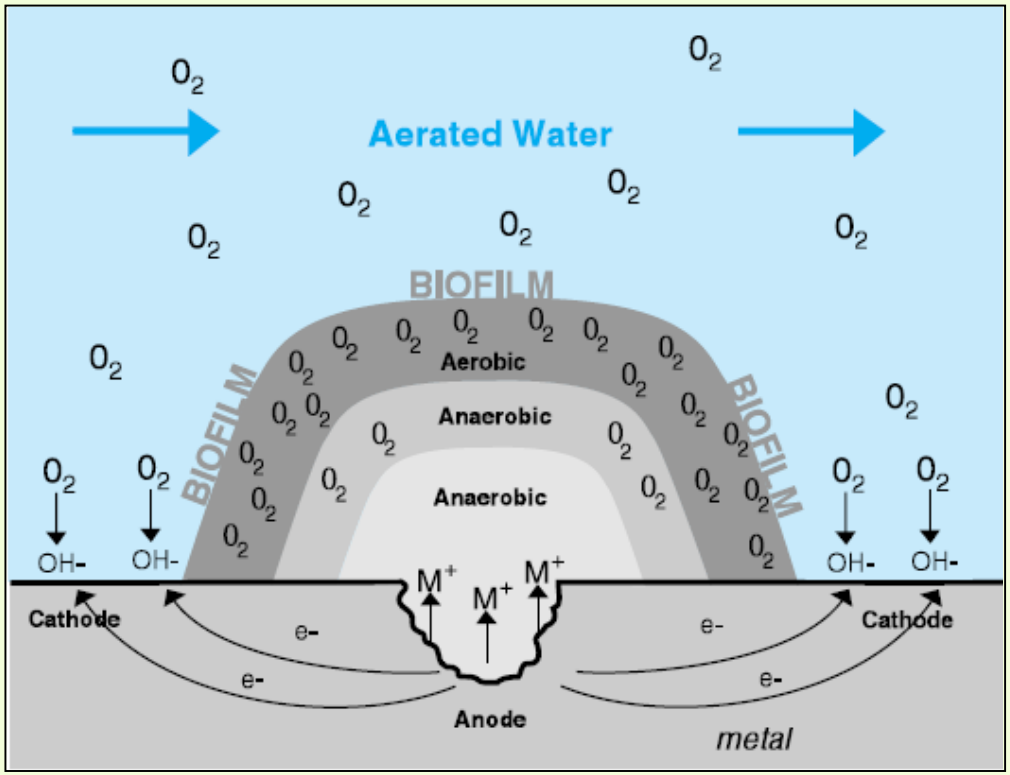
Monroe,2007;..

Biofilms: Microorganisms in the real world biofilm development



Oxygen is present at measurable concentrations mainly at the periphery of the biofilm

- Nonuniform colonization by bacteria results in differential aeration cells. This schematic shows pit initiation due to oxygen depletion under a biofilm (Borenstein,1994).



Biofilm formation

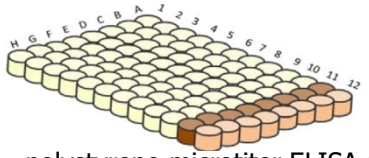
Biofilm congo red agar medium

1. The production of black colonies with a dry crystalline consistency by the organisms was taken to indicate biofilm production(positive result).
2. Non-biofilm-producing strains develop red colonies.
3. The weak slime producers usually remained pink, though an occasional darkening at the centres of the colonies was observed.

After 24 hours, the black colonies became reddish (center). However, they are still considered positive as almost black colonies, according to the colorimetric scale proposed by Arciola *et al.*, 2002. This is because low concentration of sucrose for a longer time in the culture medium (Ferreira *et al.*,2014).

Niveditha *et al.*,2013;..

Manipulation of QS



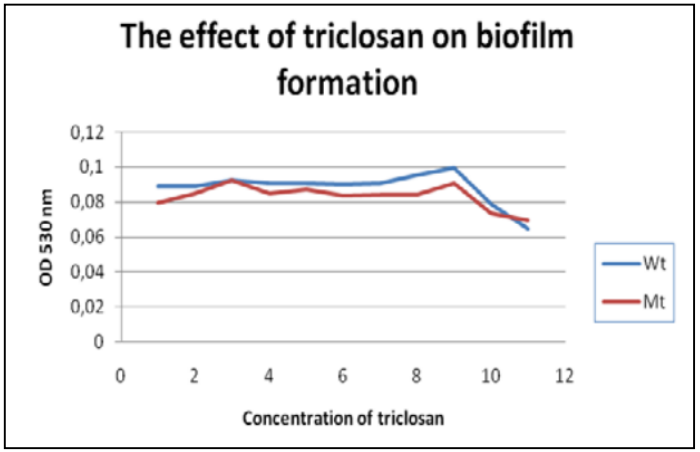
polystyrene microtiter ELISA plate

Aromatic compound disruption of bacterial biofilm

Static biofilm assay

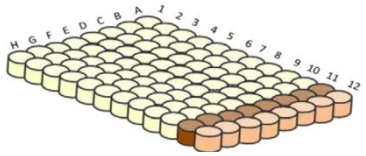
- Triclosan is a non-ionic, lipid-soluble biphenol with a broad spectrum of antibacterial, fungal and viral activity.
- Concentration of triclosan in each column on the microtiter plate used.
- Each column is represented on the x-axis on below graph.
- WT (wild-type) and MT (Mutant) of target bacterium.

Column	Concentration (µM)
1	0
2	0,509
3	0,815
4	1,304
5	2,086
6	3,338
7	5,341
8	8,544
9	13,672
10	21,875
11	35



Nguyen and Rizvi

Manipulation of QS



Polystyrene microtiter plate

Aromatic compound disruption of bacterial biofilm

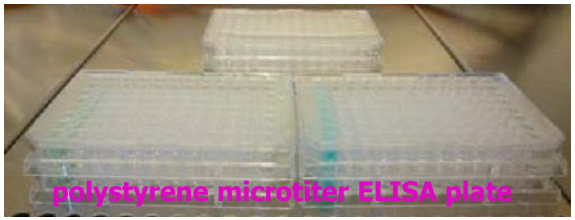
Static biofilm assay

- The OD value obtained for *Staphylococcus aureus* strains without extracts was used as the control. The reduction percentage of biofilm formation in the presence of different Juniperus extracts was calculated using the ratio between the values of $OD_{492nm}$with and without extracts, adopting the following formula:
- 100- [(OD_{492nm} with extract/OD_{492nm} without extract)x100]

ME	Initial adhesion				Biofilm formation			
	3 h		5 h		7 h		24 h	
Concentration (0.5 MIC)	$OD_{492\,nm}$	Red. %	$OD_{492\,nm}$	Red. %	$OD_{492\,nm}$	Red. %	$OD_{492\,nm}$	Red. %
Jcc	0.05 ± 0.01	44	0.08 ± 0.01	38	0.09 ± 0.03	77	0.16 ± 0.02	68
Jcs	0.07 ± 0.01	22	0.11 ± 0.01	15	0.10 ± 0.01	74	0.17 ± 0.01	66
Jd	0.07 ± 0.01	22	0.11 ± 0.01	15	0.10 ± 0.02	74	0.15 ± 0.04	70
Jom	0.06 ± 0.02	33	0.10 ± 0.01	23	0.11 ± 0.01	72	0.11 ± 0.02	78
Joo	0.05 ± 0.01	44	0.08 ± 0.02	38	0.11 ± 0.05	72	0.20 ± 0.06	60
Control	0.09 ± 0.05		0.13 ± 0.05		0.39 ± 0.16		0.55 ± 0.23	

ME, methanol extracts; Red., biofilm reduction.
The results are expressed as mean ± SDs.

Marino *et al.*,2010



Biofilm formation assay

- Biofilm formation capacity was determined macroscopically by the method of O'Toole and Kolter,1998 with some modifications.
- Sterile glass tubes/flat bottom polystyrene microtitre plates were inoculated with 800 µL bacterial culture (OD_{600} 0.5) and incubated with agitation for 72 h at 30°C.
- The planktonic cells (individual cells) were removed, and each tube was washed three times with saline solution, emptied, stained with crystal violet 0.1% for 15 min, and rinsed three times with distilled water to remove excess crystal violet. The plates were then air-dried.
- Biofilms formed were quantified by adding 1 mL 95% ethanol to the stained tube.
- The absorbance of solubilized crystal violet was determined by spectrophotometry at 570 nm.

Planktonic microorganisms might have to be increased by 10-1000 times to have the same efficacy on microorganisms in a biofilm.

Nievas *et al.*,2012; Nguyen and Rizvi

Biofilm formation

Tissue culture plate method (TCP)

Enumeration of biofilm cells

- The enumeration of viable biofilm cells on stainless steel(SS) and TCP was conducted after 24, 48, 72 and 48 hours using bead vortexing method described by Giaouris and Nychas,2006.
- Initially, the coupons were carefully removed from the petri dishes using sterile forceps, rinsed twice by pipetting with 10 ml of PBS, with shaking in order to remove the loosely attached cells.
- After the second rinsing step, each coupon was individually transferred into 50 ml plastic tube containing 10 ml physiological saline (0.95% NaCl w/v).

Muazu *et al.*,2013

Biofilm formation

Tissue culture plate method (TCP)

Enumeration of biofilm cells

- The plastic tube was vortexed for 2 min at maximum speed to detach biofilm cells from the coupons.
- Detached cells were subsequently enumerated by agar plating.
- Ten-fold of six serial dilutions were made and 100 µl from the sixth dilution was pipetted onto Tryptic Soy Broth and incubated at 37°C for 24 hours.
- Finally plates were removed after 24 hours of incubation.
- Developed colonies were counted taking a range of 3-300 while viable cells were expressed colony forming unit per mill (CFU/ml).
- The experiments were repeated three times.

Muazu *et al.*,2013

Biofilm formation
Coaggregation and autoaggregation

- **Coaggregation:**
- In coaggregation, the genetically distinct bacteria specifically recognize one another, may contribute to the retention and enrichment of different species within these biofilms.

- **Autoaggregation:**
- Autoaggregation (self-aggregation) is distinct from autoaggregation, which is the recognition and adhesion of genetically identical bacteria.

Autoaggregation was scored by using the same criteria as those used for coaggregation.

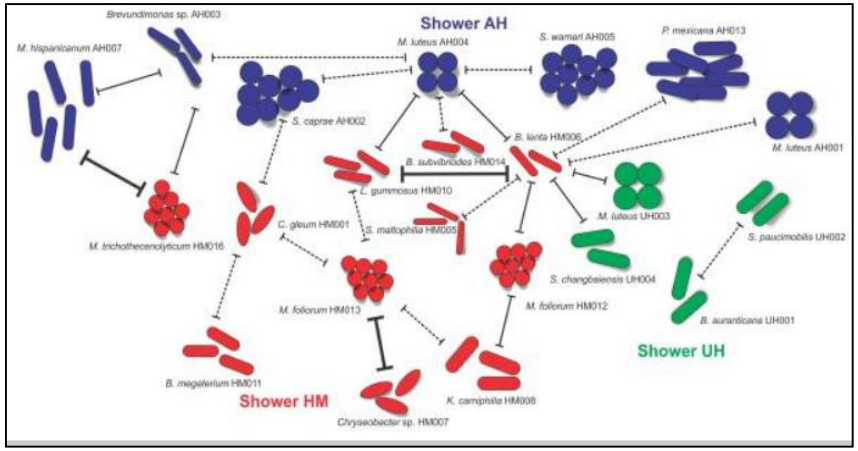
Vornhagen *et al.*,2013

Biofilm formation

The role of coaggregation in biofilm formation

Inter- and intra-biofilm coaggregation ability of each culturable isolate

- **Intra-and inter-biofilm quorum sensing communication.** Coaggregation partnerships between isolates from within the same shower biofilm (intra-biofilm coaggregation) and with isolates from different shower biofilms (inter-biofilm coaggregation).



A diagrammatic representation of the inter- and intra-biofilm specificity of coaggregation between the showerhead biofilm isolates after growth in batch culture for 48h. Visual coaggregation scores <2 are not shown. Colors highlight the showerhead from which the isolates were actually harvested. Cells are not to scale and visual scores are depicted as connecting lines of different thickness. Thickest line (—) represents a visual coaggregation score of 4, line of intermediate thickness represents score of 3 (—), and thinnest dotted line (----) represents a score of 2.

Vornhagen *et al.*,2013;..

Biofilm formation

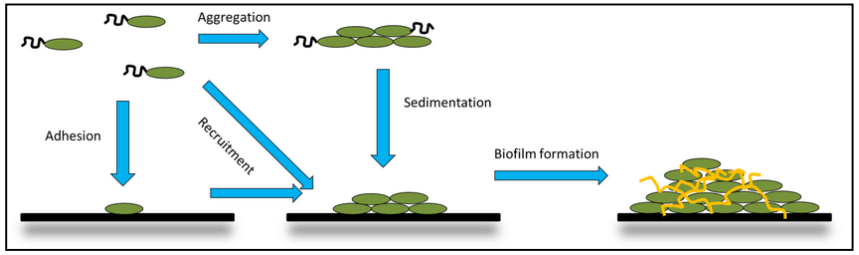
Microbial cells show two growth modes either as planktonic cells(individual cells) or as biofilms

The role of autoaggregation in biofilm formation

- Autoaggregation can lead to biofilm formation in two ways:

1. planktonic cells (individual cells) can either attach to a substrate surface as single cells and then recruit (join) more planktonic cells via aggregation to form a single microcolony, or
2. planktonic cells aggregate in suspension and then settle on the substrate surface.

- Both pathways can lead to the formation of biofilm.



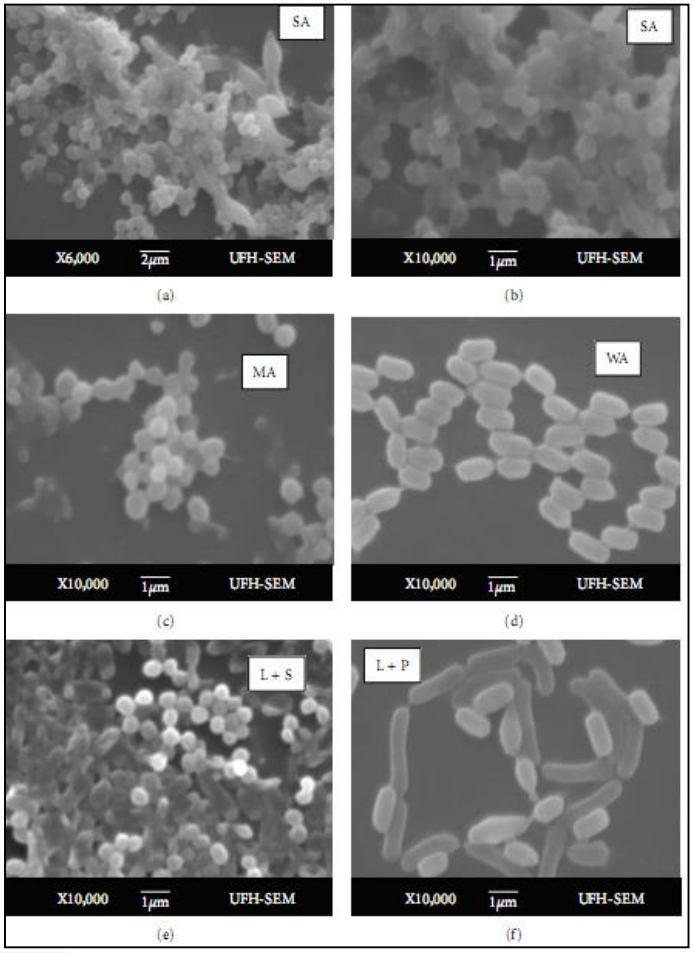
Biofilm formation involves several steps which start with the attachment of planktonic cells to an inert or biological surface, followed by the growth of colonies developing three dimensional structures, and the potential detachment of colonies as the last phase.

Trunk *et al.*,2018;..

Biofilm formation

Coaggregation and autoaggregation assays

- Scanning electron micrographs of autoaggregates and coaggregates biofilms of *Listeria ivanovii* and their coaggregates partner *Staphylococcus aureus* NCTC 6571 and *P. aeruginosa* ATCC 15442.
- SA, strong adherent autoaggregate;
- MA, moderate adherent autoaggregate;
- WA, weak adherent autoaggregate;
- L + S, coaggregates of *L. ivanovii* and *S. aureus*;
- L + P, coaggregates of weak adherent *L. ivanovii* and *P. aeruginosa*.



Trunk *et al.*,2018

Biofilm formation

Coaggregation and autoaggregation assays

- **Composition of Brain Heart Infusion (BHI) Broth.**
- The medium contains proteose peptone and infusions from calf brain and beef heart which serve as sources of carbon, nitrogen, essential growth factors, amino acids and vitamins. Dextrose is used as a source of energy. Disodium phosphate helps in maintaining the buffering action of the medium whereas sodium chloride maintains the osmotic equilibrium of the medium.

Ingredients	Gms / Litre
Calf brain, infusion from	200
Beef heart, infusion from	250
Proteose peptone	10
Dextrose	2
Sodium chloride	5
Disodium phosphate	2.5
Final pH (at 25°C)	0.2±7.4

BHI maintains a wide variety of fastidious and non-fastidious microorganisms, including aerobic and anaerobic bacteria. For environmental factors, amylase, calcium ion, and magnesium ion significantly increased both autoaggregation and coaggregation ability, while these abilities were significantly decreased by lactose.

microbeonline.co; Plansangket *et al.*,2019

Biofilm formation

Coaggregation and autoaggregation assays

- Twelve (three each nonadherent, weakly adherent, moderately adherent, and strongly adherent) *L. ivanovii* isolates and seven reference strains (*S. aureus* , *S. pyogenes* , *S.* Typhimurium, *P. aeruginosa*, *P. shigelloides*, *A. hydrophila*, and *S. sonnei*) were used for these assays.
- The bacteria strains were grown separately in 20 mL of Brain Heart Infusion Broth (BHI) broth at 37°C for 48 hours. Cells were harvested by high-speed centrifugation (11,000 ×g for 10 min) and washed twice in 3 mM NaCl containing 0.5 mM $CaCl_2$. Subsequently, the cells were resuspended in the same solution (3 mM NaCl containing 0.5 mM $CaCl_2$) and centrifuged at 650 ×g for 2 min, and the supernatant carefully aspirated and discarded into a waste container.
- The OD of the cell suspension was measured and adjusted to 0.3 using an automated spectrophotometer at a wavelength of 660 nm; the cell suspension was used for coaggregation assay.
- Equal volumes (1 mL each) of the coaggregating partners were mixed and the OD (OD_{Tot}) of the mixture was immediately read at 660 nm before incubation at room temperature for 2 hours. Subsequently, the tubes were centrifuged at 2,000 rpm for 2 min and the OD of the supernatant (OD_s) measured at the same wavelength (660 nm).

Trunk *et al.*,2018

Biofilm formation

Coaggregation and autoaggregation assays

- The degree of coaggregation of the paired isolates was determined using the equation:

$$\% \text{ coaggregation} = \frac{OD_{Tot} - OD_s}{OD_{Tot}} \times 100.$$

OD_{Tot} value refers to the initial OD immediately after the pairing of isolates and OD_S denotes the OD of supernatant after 5 hours at room temperature.

- For autoaggregation assay, the individual bacterial suspension adjusted to an OD of 0.3 was incubated at room temperature for 1 hour and the cell suspension centrifuged at 2000 rpm for 2 minutes. The supernatant (2 mL) was transferred into a cuvette and the OD measured at 660 nm.
- The degree of autoaggregation was calculated as follows:

$$\% \text{ autoaggregation} = \frac{OD_0 - OD_{60}}{OD_0} \times 100.$$

- OD_0 refers to the initial OD of the organism, and OD_{60} is the OD of the supernatant after 60 min of incubation.

Trunk *et al.*,2018;..

Biofilm formation
Microtiter Adherence Assay

- **The biofilm formation ability of 50 *Listeria ivanovii* strains:**
- Variations in biofilm formation were observed.
- A total of 44 (88%) strains adhered to the wells of the microtiter plate while 6 (12%) did not adhere.
- The majority of the isolates demonstrated weak (44%) and moderate (34%) adherence while only 5 (10%) strains strongly adhered to the wells.
- The optical density range of nonadherent and strong adherent isolates was 0.332–0.503 and 2.32–3.846, respectively.

Biofilm formation by *Listeria ivanovii* isolates (*n*=50) following incubation at 25°C.

Biofilm formation	Number (%)	OD Range	Mean OD ± SD
Nonadherent	6 (12)	0.332–0.503	0.431 ± 0.055
Weak adherent	22 (44)	0.545–1.083	0.785 ± 0.175
Moderate adherent	17 (34)	1.105–2.084	1.432 ± 0.354
Strong adherent	5 (10)	2.32–3.846	3.045 ± 0.887
Total biofilm	44 (88)	0.545–3.846	1.754 ± 0.763

D: optical density; SD: standard deviation. The results are the mean of three independent experiments carried out in triplicates.

Trunk *et al.*,2018

Biofilm formation

Coaggregation and autoaggregation assays

- Coaggregation occurred to varying degrees between all the seven partner strains and *L. ivanovii* isolates.
- The coaggregation index ranged from 12 to 77% while autoaggregation ranged from 11 to 55%.
- Some strains which strongly adhere to the wells were equally able to stick to each other (autoaggregation of 32-55%); this was followed by moderate (35-41%) and weak adherent (30-46%) strains while nonadherent cells registered the least autoaggregation 11-20%.
- On the other hand, moderate adherent strains had a slightly high coaggregation index range of 44-77% followed by strong adherent 41-77% and nonadherent 12-40% strains.
- It was also observed that 95% of the moderately adherent strains had a coaggregation index of >50% while the weak and strong adherent strains had 90% each.

Biofilm phenotype	% Autoaggregation range	% Coaggregation range	Coaggregation indices > 50%
Non adherent	11–20	12–40	0
Weak adherent	30–46	37–75	90
Moderate adherent	35–41	44–77	95
Strong adherent	32–55	41–77	90

Nyenje *et al.*,2012

Biofilm formation

Coaggregation and autoaggregation assays

- % Auto-aggregation = $(1 - (A_t / A_{initial}) \times 100$. where A_t and $A_{initial}$ represent the absorbance at time t (i.e., 0.5, 1, 2, 3, 4, and 5 hours) and time 0, respectively.
- The co-aggregation of the isolated strains with 6 pathogens was also determined similar to autoaggregation.
- Briefly, the same volume of each *Lactobacillus* and pathogen strains were mixed and incubated at room temperature for 5 hours and the co-aggregation percentages were monitored after incubation according to the previously described method(Khan and Kang,2016).
- The co-aggregation was calculated using the following equation:
- %Co-aggregation= $((OD_{Tot} - OD_S)/ OD_{Tot}) \times 100$.
- OD_{Tot} value refers to the initial OD immediately after the pairing of isolates and OD_S denotes the OD of supernatant after 5 hours at room temperature.

Zangeneh *et al.*,2019

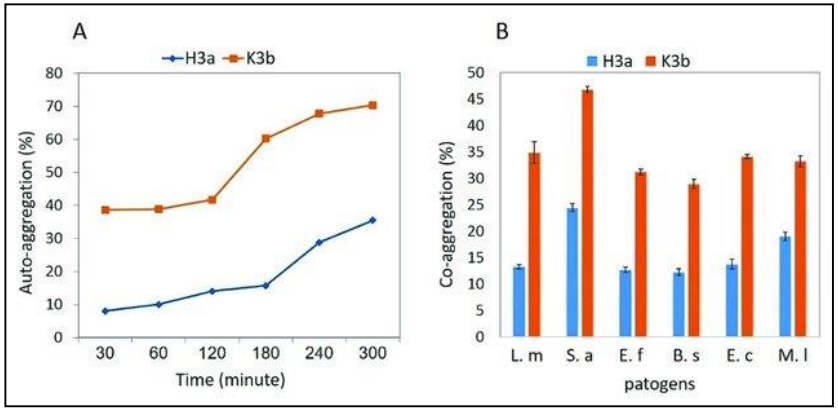
Biofilm formation

Coaggregation and autoaggregation assays

A. Auto-aggregation of *Lactobacillus* strains H3a and K3b and in a 5-hour period;

B. Co-aggregation percent of strains with different pathogens.

- Note:
- L.m: *Listeria monocytogenes*;
- S.a: *Staphylococcus aureus*;
- E.f: *Enterococcus faecalis*;
- B.S: *B. subtilis*;
- E.c: *E. coli*;
- M.l: *Micrococcus luteus*.



***Lactobacillus* strains H3a and K3b showing antibacterial/antagonistic activity against 6 pathogens of human gut.**

Slide A, the autoaggregation of K3b (72%) during 5 hours of incubation at room temperature was significantly more than that of H3a (39%).

Slide B, the co-aggregate assessment results revealed that the ability of the coaggregation of K3b with pathogens was more than that of H3a.

Further, the K3b strain showed the highest rate of co-aggregation with *S. aureus* PTCC1112.

Zangeneh *et al.*,2019

Biofilm and Autoaggregation Assays

- Bacterial biofilms are extremely heterogeneous, both:

1. structurally (comprising different populations of microorganisms), and
2. in the physiology of the bacterial cells within them.

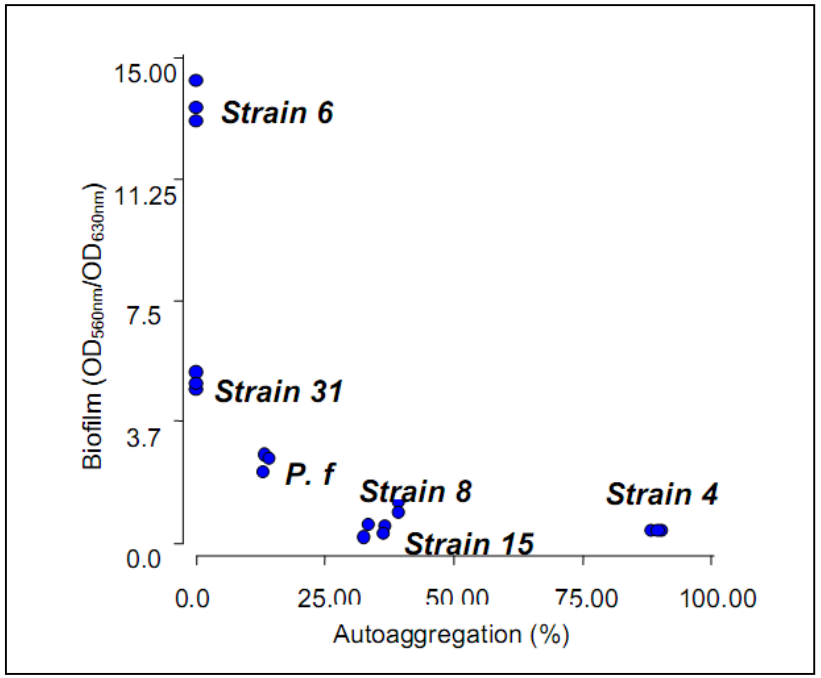
- The autoaggregation of bacteria behaved similarly to biofilm formation, in that the heterogeneity was quite high; while some strains strongly autoaggregated, others did not.

Isolate	Biofilm (OD_{560nm}/OD_{630nm})	Autoaggregation (%)
E4	0.38 ± 0.02	89.41 ± 1.08
E6	13.58 ± 0.62	0
E8	0.44 ± 0.23	34.16 ± 2.33
S15	0.86 ± 0.52	38.14 ± 1.55
SE31	5.00 ± 0.26	0
PF	2.51 ± 0.26	13.54 ± 0.55

López *et al.*,2018

Biofilm and Autoaggregation Assays

- We performed a correlation analysis to determine whether the ability of the strains to autoaggregate and form biofilm was quantitatively related.
- A scatter plot was generated and the Pearson correlation coefficient (r) was calculated.
- We observed an inverse correlation between both phenotypes ($r \leqslant -0.64$, $p \geq 0.05$), maybe because the cell interactions of the biofilm formation and aggregates were not determined equally on the same physical adhesive forces.



Scatter plot of two variables: biofilm formation ability (OD_{560nm}/OD_{630nm}) and autoaggregation (%). The Pearson correlation coefficient (r) was calculated using Infostat, version 1.0.

López *et al.*,2018

Biofilm and Autoaggregation Assays

- **Autoaggregation assay:**
- Bacteria were grown in 2 mL nutrient broth at 28°C for 24 h, diluted 1/100 in nutrient broth and incubated for 48 h under the same conditions. Bacterial suspensions (5 mL) were then transferred into a glass tube and allowed to settle for 24 h at 4°C.
- The optical density of these suspensions at 630 nm (OD_{final}) was measured.
- A control tube was vortexed for 30 s and the initial OD_{630nm} (OD_{inital}) was determined.
- The percentage of autoaggregation was calculated as follows: $100 \times [1- (OD_{final}/OD_{initial})]$.

López *et al.*,2018

Biofilm and Autoaggregation Assays
Autoaggregation assay

- The bacteria were grown in 2 mL TY medium supplemented with appropriate antibiotic, incubated for 24 h at 30°C, diluted 1/100 in TY or MGM low phosphate medium, and incubated for 48 h under the same conditions.
- The bacterial suspensions (5 mL) were then transferred to a glass tube (10 x 70 mm) and allowed to settle for 24 h at 4°C.
- A 0.2 mL aliquot of the upper portion of the suspension was transferred to a microtiter plate, and OD_{600} was measured (OD_{final}).
- A control tube was vortexed for 30s, and OD_{600} was determined ($OD_{initial}$).
- The autoaggregation percentage was calculated as $100[1-(OD_{final}/OD_{initial})]$.
- For both homologous and heterologous autoaggregation assays, cultures were centrifuged at 4200 x g (5847 rpm)for 20 min prior to the settling period.

1. For homologous assay, the pellet of a given strain was resuspended in cell-free supernatant from an independent culture of the same strain.
2. For heterologous assay, the pellet was resuspended in cell-free supernatant from a culture of a different strain.

Sorroche *et al.*,2012

Anti-biofilm formation assay

1. The efficacy of ZnO NPs on un-formed biofilm:

- Different concentrations (100, 200, 300, 400, 500, 600 and 700 mg/100 ml) of zinc oxide nanoparticles (ZnO NPs) were prepared by suspending the nanoparticles in double-distilled water.
- Individual wells of sterile, polystyrene, 96-well-flat bottom tissue culture plates (TCP) were filled with 180 µl of Brain Heart Infusion Broth (BHI) and inoculated with 10 µl of overnight culture (OD_{620}=0.01). Immediately after vigorous vortex mixing, 10 µl of ZnO NPs were added from the stocks to the wells, so that final concentrations were made between 50 and 350 µg/ml.
- Final volume in every well was 200 µl.
- The tissue culture plates were incubated for 24 h at 37°C. After incubation, content of each well was gently removed. Then (next slide)

Sangani *et al.*,2015

Anti-biofilm formation assay

- The wells were washed four times with phosphate buffered saline solution (pH=7-7.2) to remove free-floating planktonic bacteria.
- Biofilms formed by bacteria were fixed with ethanol (95%) and stained with crystal violet (0.1%, w/v).
- Excess stain was rinsed off by several times washing with deionized water and plates were kept for drying.
- 0.225 ml of glacial acetic acid (33%) was added to the wells to release he bound dye from the stained cells.
- Biofilm formation was quantified by measuring the OD_{590} of the solution with a microplate reader.
- These OD values were considered as an index of bacteria adhering to surface and forming biofilms. Experiments were performed in triplicate, the data was then averaged.

Glacial acetic acid is a name for water-free (anhydrous) acetic acid.

Sangani *et al.*,2015

Anti-biofilm formation assay

2. The efficacy of ZnO NPs on formed biofilm:

- To evaluate the effect of ZnO NPs on elimination of pre-formed biofilm, individual wells of TCP were filled with 180 µl of BHI broth and inoculated with 10 µl of bacterial suspension (OD_{585} = 0.01).
- The tissue culture plates were incubated for 24 h at 37°C.
- After incubation, 10 µl of ZnO NPs dilutions were added to each well. So that final concentration of ZnO in each well became 50 to 350 µg/ml. A well containing 0.2 ml of BHI broth inoculated with bacteria was considered as positive control. After 2 h, the content of the micro plate was gently removed and as described earlier, OD of stained adherent bacteria in wells were read at 590 nm.

Sangani *et al.*,2015

Anti-biofilm formation assay

- The percentage of inhibition was calculated by the formula as:

$$\frac{control\ OD585\ nm - test\ OD585\ nm}{control\ OD585\ nm} \times 100$$

Venkadesaperumal *et al.*,2016

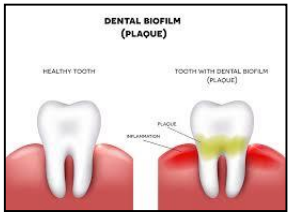
Biofilm

Useful vs. harmful biofilms

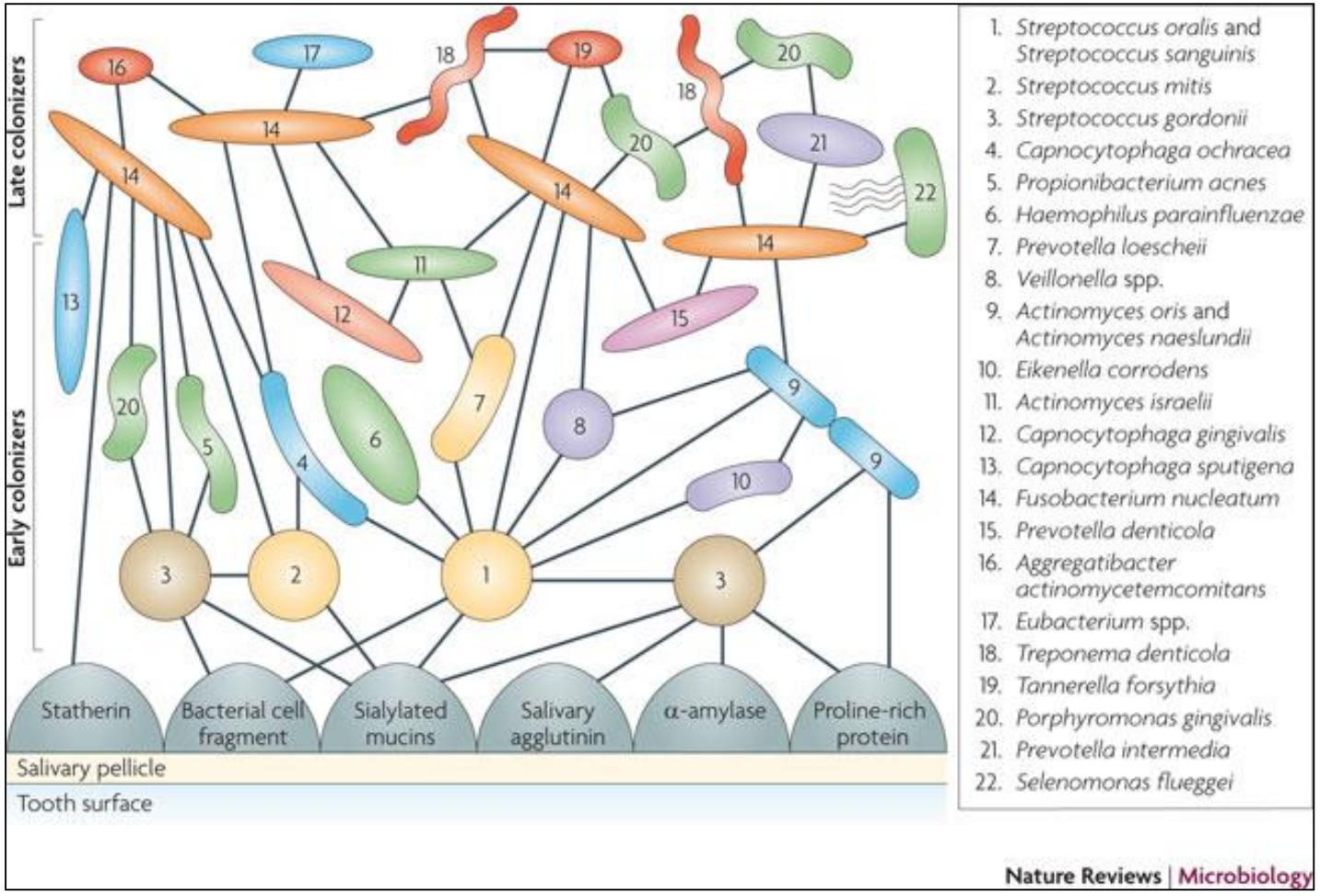
- Biofilms are ubiquitous in nature and can be harmful in some cases and beneficial in others.
- e.g. the rapid formation of useful biofilms, such as biofilters to clean water or biobarriers to contain contamination, might be encouraged now that biologists to know more about the quorum-sensing signal/ biofilms.
- A deeper understanding of signaling could improve our ability to intervene in these and other processes.

Biofilms

Dental biofilm(plaque)



Bacterial species involved with dental biofilm accumulation and tooth decay. All employ quorum sensing.



Giuliano,2013

Biofilm management

Dental biofilm(plaque)

Acylase I quench the biofilm formation

- Acylase I to quench the biofilm formation by environmental strains of bacteria.
- Acylase I was found to reduce biofilm formation by *Aeromonas hydrophila* and *P. putida* on:
- borosilicate membrane, made up of silica (36% and 23%, respectively),
- polystyrene membrane (60% and 73%, respectively) and a
- reverse osmosis membrane (20% and 24%, respectively).

Borosilicate glass (pyrex) is a type of glass with silica. polystyrene is a synthetic resin which is a polymer of styrene, used chiefly as lightweight rigid foams and films. Reverse osmosis (RO) is a water purification technology that uses a semipermeable membrane to remove larger particles from drinking water.

Biofilm management

Dental biofilm(plaque)

Biotene PBF mouthwash

- Normal mouthwashes can only clean the surface, which is why bad breath returns quickly and gum disease is a constant problem.
- With the new patented technology in Biotene PBF mouthwash, you can dissolve the biofilm, expose hidden bacteria colonies and kill germs.
- In addition, Biotene PBF contains the proven LP3 salivary enzyme system to strengthen the body's antibacterial action, dissolving biofilm and inhibiting excessive bacteria – maintaining a healthy oral balance."

Usman and Lalama

Biofilm management

- The slow metabolism of biofilm cells also contributes to their resistance.
- The bacteria in the film are relatively quiescent (inactive) and divide only rarely.
- Antibiotics such as the penicillins, which need to be incorporated in the cell wall, are only effective against actively dividing cells.
- However, other antibiotics work just as well against quiescent cells, because they target basic cellular processes such as metabolism or protein or DNA synthesis.
- For reasons that are still being clarified, even these antibiotics are less effective against biofilms.

Monroe,2007

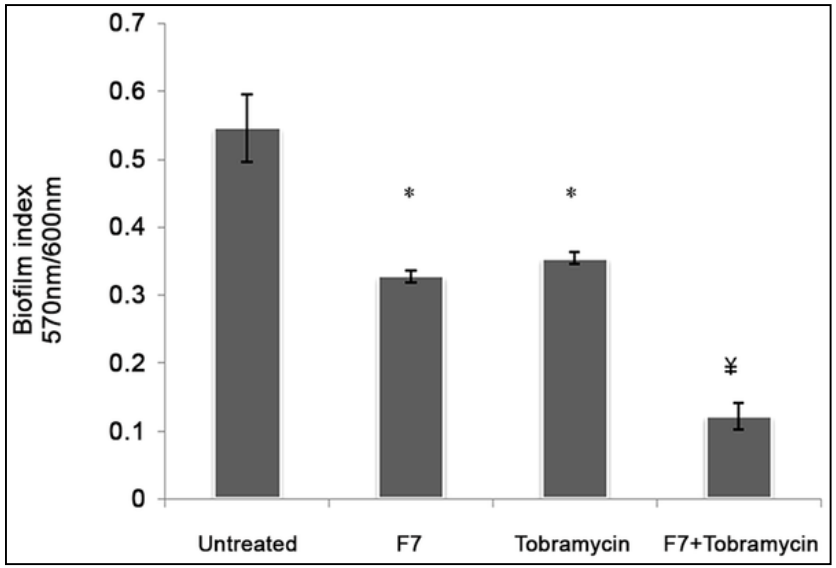
Biofilm management

Plant extract and antibiotic treatments

Biofilm of *P. aeruginosa* PAO1

Terminalia chebula is a species of Terminalia, native to southern Asia.

- Effect of F7 (1 mg/ml) and antibiotic tobramycin (20 µg/ml) on biofilm formation by *P. aeruginosa* PAO1.
- F7 is bioactive fraction of *Terminalia chebula* obtained by fractionation of methanol extract using Sephadex LH20.

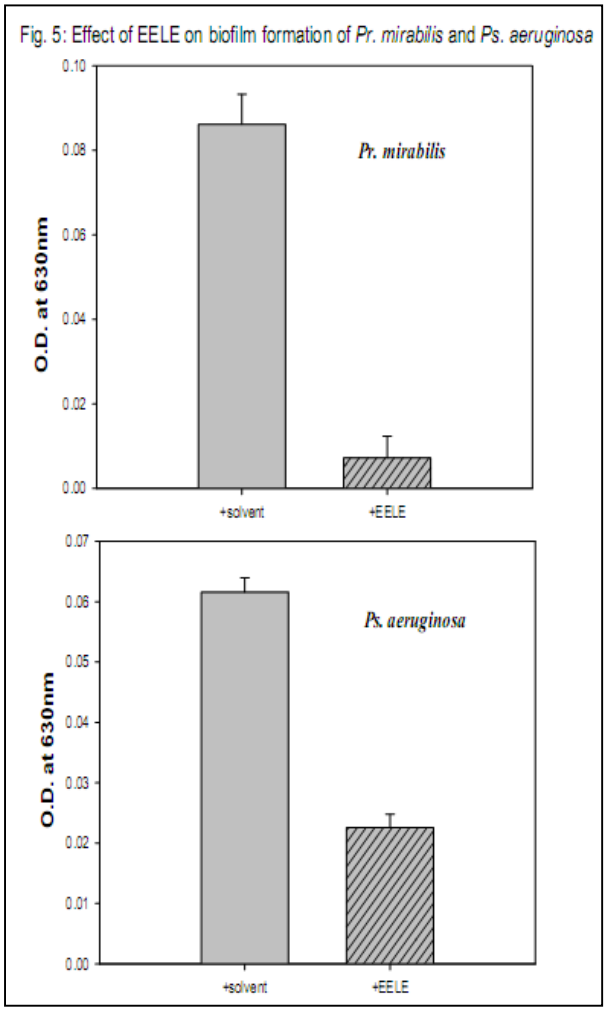


Sarabhai *et al.*,2013

Biofilm management

Plant extracts treatments

- The assay was performed using the microtiter plate method in the presence of euphorbia (spurge) ethanolic latex extract, EELE (50 µlml^{-1}) for both *Proteus mirabilis* and *Ps. aeruginosa*.
- Wells containing an equal volume of 20% DMSO served as solvent control.



Nashikkar *et al.*,2011

Biofilm management

A combination of the two treatments

Synergistic activity

- Crude plant extracts and antibiotic compounds:
- Individual plant extracts increased activity of some antibiotics while decreasing the activity of others.
- For instance, the methanolic plant extracts from Euphorbia species(*Euphorbia macroclada*) antagonized penicillin G and nalidixic acid activity, allowing over 100% percent bacterial growth.
- In contrast, tetracycline inhibition was augmented and only 26.9% bacterial growth was observed.
- Likewise, the significant effects of peppermint (*Mentha piperita*)and tea (*Thea sinensis*) extracts increasing tetracycline inhibitory activity were observed.

Nagy,2010

Biofilm management

A combination of the two treatments

Synergistic activity

- Crude plant extracts and antibiotic compounds:
- Nascimento *et al.*,2000 demonstrated that a number of crude plant extracts, including thyme, showed an increased killing effect against *Pseudomonas* species when combined with ineffective dosages of commercial antibiotics.
- For example, the plant extract of *Thymus vulgaris* can inhibit *Pseudomonas* growth at 70 µg/mL; but when combined with ineffective doses (50 µg/mL) of tetracycline, the amount of thyme needed to produce growth inhibition was reduced to 10 µg/mL.

Nagy,2010

Biofilm management

A combination of the two treatments

Synergistic activity

- Crude plant extracts and antibiotic compounds:
- It happens in two process:

1. In thyme-tetracycline therapy against antibiotic and non-antibiotic resistant strains of *P. auroginosa*, thymol appears to prevent MexAB-OprM efflux pump gene expression.
2. By blocking MexAB-OprM expression, tetracycline antibiotic accumulation can occur within the cell, thus allowing bacterial cellular damage.

Nagy,2010

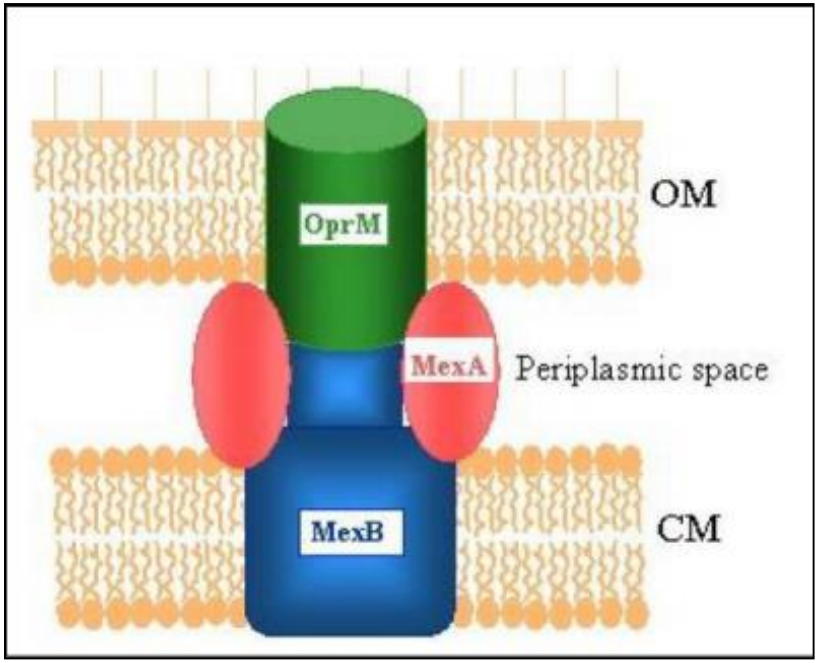
MexAB-OprM multidrug efflux pump

1)Thyme-tetracycline therapy block MexAB-OprM expression, 2)tetracycline antibiotic accumulation can occur within the cell,3) bacterial cellular damage

- Efflux-pumps are present in all organisms.
- These efflux pumps can remove (expel) antibiotics, dyes, detergents, solvents and other compounds from the cell e.g. bacterial cell before they can cause harm.
- There are two types of efflux pumps that are seen in bacteria:

1. Those which are encoded in the chromosome and
2. Those which are plasmid borne.

- MexAB-OprM is under quorum sensing control.



OM: outer membrane, CM: cytoplasmic or inner membrane.

Nagy,2010

Biofilm management

Bacteriophage treatments

- Numerous bacteriophages of bacterial plant pathogens have been described in the past (Okabe and Goto,1963) including phages of *R. solanacearum* (Hayward,1964)and its banana-attacking strain.
- At least one of these phages has been found to produce a bacteriolytic protein against *R. solanacearum*.

Biofilm management
Bacteriophage treatments

- EPS and biofilms render many bacteria resistant to treatment and environmental perturbations.
- One of the most exciting areas of research is the use of bacteriophage to penetrate biofilms and directly attack pathogens.
- Bacteriophages are natural predators of bacteria, and some carry genes that enable them to effectively depolymerize biofilms.

Biofilm management

Bacteriophage treatments

- An attractive way to understand the role of EPS in more depth and eventually increase disease resistance by reduced EPS production in plant pathogenic bacteria could be through the use of polysaccharide depolymerase enzymes.
- Hartung *et al.*,1986 described the isolation of a polysaccharide depolymerase gene from a bacteriophage of *E. amylovora*.
- The purified recombinant enzyme degraded amylovoran, the acidic component of the EPS abolished the virulence of *E. amylovora* in bioassays, and inhibited bacterial cell growth.

Biofilm management
Bacteriophage treatments

- These observations indicate that correct expression of the gene in plants may be useful for testing this approach to control bacterial diseases.
- These bacteriophages apparently use additional ways to facilitate the infection of their host (bacteria), which includes a lysozyme and a holin that may form a pore to support cell lysis by the lysozyme.

Holins (from making holes) are proteins act by permeabilizing the host cell cytoplasmic membrane.

Biofilm management

Different functions of salicylic acid on biofilm formation by plant pathogenic bacteria

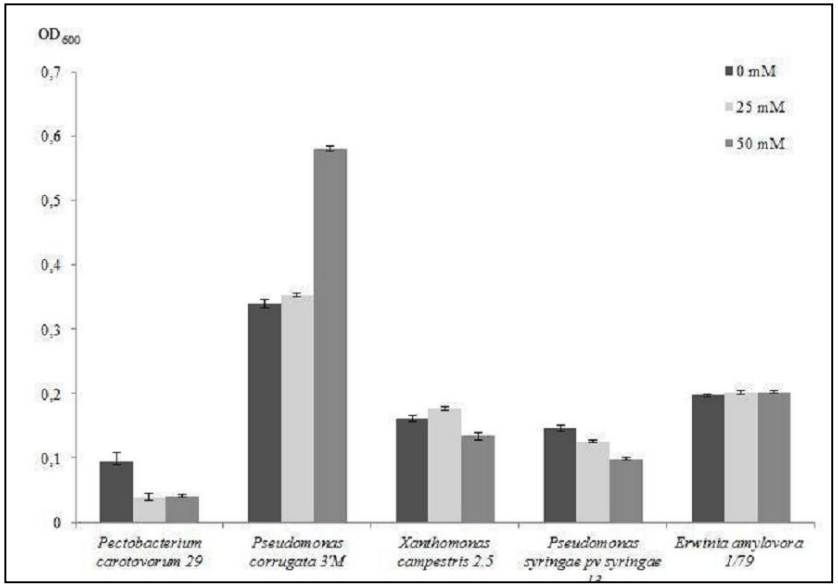
- Salicylic acid (SA) is an important plant hormone whose concentration in plants is regulated by abiotic and biotic factors.
- It's a well-known fact that microbial infection induces accumulation of endogenous SA.
- As a result, the systemic acquired resistance occurs.
- We suggested that SA not only triggers plant defense responses but also directly affects production of virulence factors by plant pathogenic bacteria.
- SA is as an antimicrobial agent that directly affects cells of plant pathogenic bacteria.

Lagonenko *et al.*,2013

Salicylic acid

Biofilm formation by different phytopathogenic bacteria in presence of SA

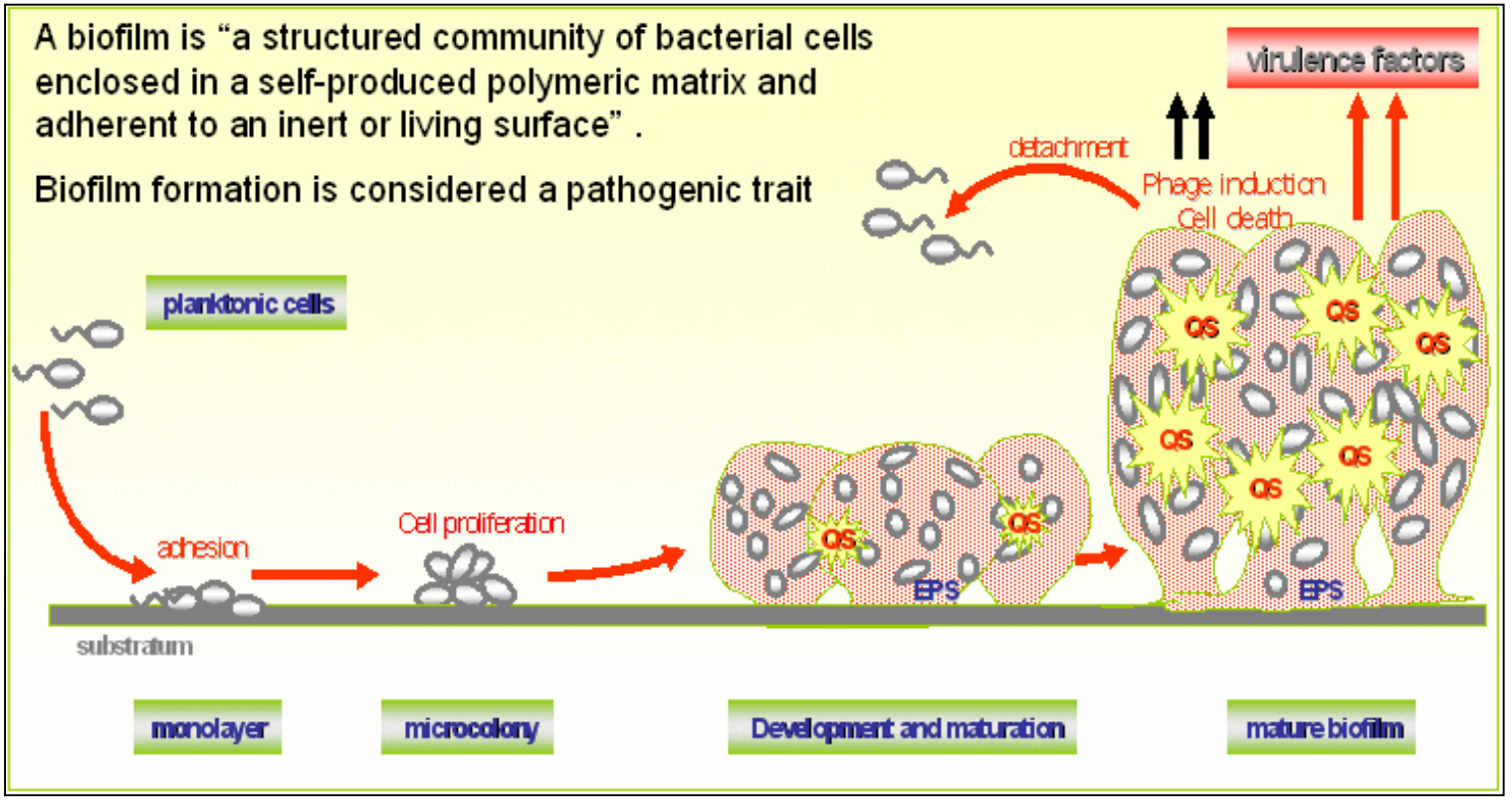
1. SA induces biofilm formation by *Pseudomonas corrugata*.
2. Whereas, SA inhibits biofilm formation by *Pectobacterium carotovorum* and *Pseudomonas syringae* pv. *syringae* at sub inhibitory concentrations.



Lagonenko *et al.*,2013

Biofilm management

Bacteriophage treatments



Biofilm management

The seaweed *Delisea pulchra*

Biosignal's anti-biofilm technology

- Biosignal's anti-biofilm technology is based on the discovery that the red alga *Delisea pulchra* produces natural furanones that can disable bacteria's ability to colonize.
- **About the Product**
- **Company:** (formerly) BioSignal, Inc.
  Product Phase: Under development.
  Product Type: Biofilm control technology.
  Patent Name: Association of antimicrobial compounds with surfaces and polymers.
  Patent Number: WO 2005/053684 A1.

Marine algae, more commonly known as seaweeds.
Furanones chemical structures similar to the *N*-acylhomoserine lactones may inhibit biofilm formation through interference with quorum sensing(QS signal-mimics).

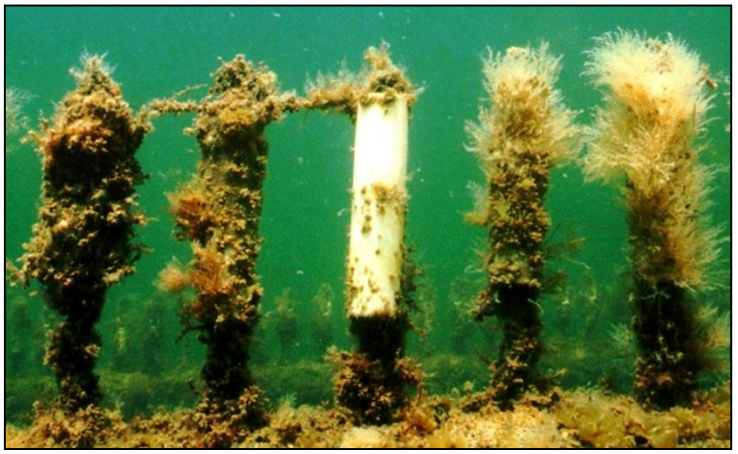
Ciba Specialty Chemicals

Biofilm management

The seaweed(red alga) *Delisea pulchra*

Biosignal's anti-biofilm technology

- Marine fouling is a major problem for waterborne craft around the world and results in significant additional fuel and maintenance costs for operators.
- BioSignal Ltd. is now testing and/or already applying synthetic furanones based on those produced by *Delisea pulchra* in a variety of applications.



Delisea pulchra protects itself from bacterial infection by exuding compounds that inhibit biofilms (middle image).

Fouling is the accumulation of unwanted material on solid surfaces to the detriment of function.

Ciba Specialty Chemicals

Biofilm management

BioFilm, Inc, a privately held company

- BioFilm, Inc, a privately held company, founded in 1991, manufactures and distributes high quality healthcare products that contribute to the well being of all who use them.
- Our flagship product, Astroglide Personal Lubricant, is one of the world's top selling personal lubricants.

Biofilm

Lyme disease (*Borrelia burgdorferi*) infections and biofilm

- The morphologic diversity of Borrelia within biofilms (cyst, granular, L form, spiral forms, etc.), together with a variety of concurrent co-infections, makes it almost impossible for the body to eliminate the microbes on its own, lengthening the suffering and anguish of Lyme patients.