Agrobacterium and Ti plasmids - a brief history

Agrobacterium tumefaciens - the causative agent of crown gall disease

Crown gall tumors:

- Collections of cells growing in an undifferentiated, uncontrolled manner (a tumor)
- Occur usually at wound sites
- Can be grown axenically as callus cultures -> exposure to Agrobacterium produces a heritable (at least through mitosis) change in plant cells in the tumor
- Sterile crown gall cultures do not require phytohormones (contrast with normal plant cell cultures) -> crown gall cells make cytokinins and auxins
- Crown gall cells produce large amounts of opines (amino acid and/or sugar derivatives)

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MICROBIOLOGY

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Historical account on gaining insights on the mechanism of crown gall tumorigenesis induced by *Agrobacterium tumefaciens*

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The plant tumor disease known as crown gall was not called by that name until more recent times. Galls on plants were described by Malpighi (1679) who believed that these extraordinary growth are spontaneously produced. *Agrobacterium* was first isolated from tumors in 1897 by Fridiano Cavara in Napoli, Italy. After this bacterium was recognized to be the cause of crown gall disease, questions were raised on the mechanism by which it caused tumors on a variety of plants. Numerous very detailed studies led to the identification of *Agrobacterium tumefaciens* as the causal bacterium that cleverly transferred a genetic principle to plant host cells and integrated it into their chromosomes. Such studies have led to a variety of sophisticated mechanisms used by this organism to aid in its survival against competing microorganisms. Knowledge gained from these fundamental discoveries has opened many avenues for researchers to examine their primary organisms of study for similar mechanisms of pathogenesis in both plants and animals. These discoveries also advanced the genetic engineering of domesticated plants for improved food and fiber.

Keywords: Ti plasmid, *Agrobacterium*, T pilus, T-DNA, type IV secretion system, type VI secretion system, opines, conjugative transfer

INTRODUCTION

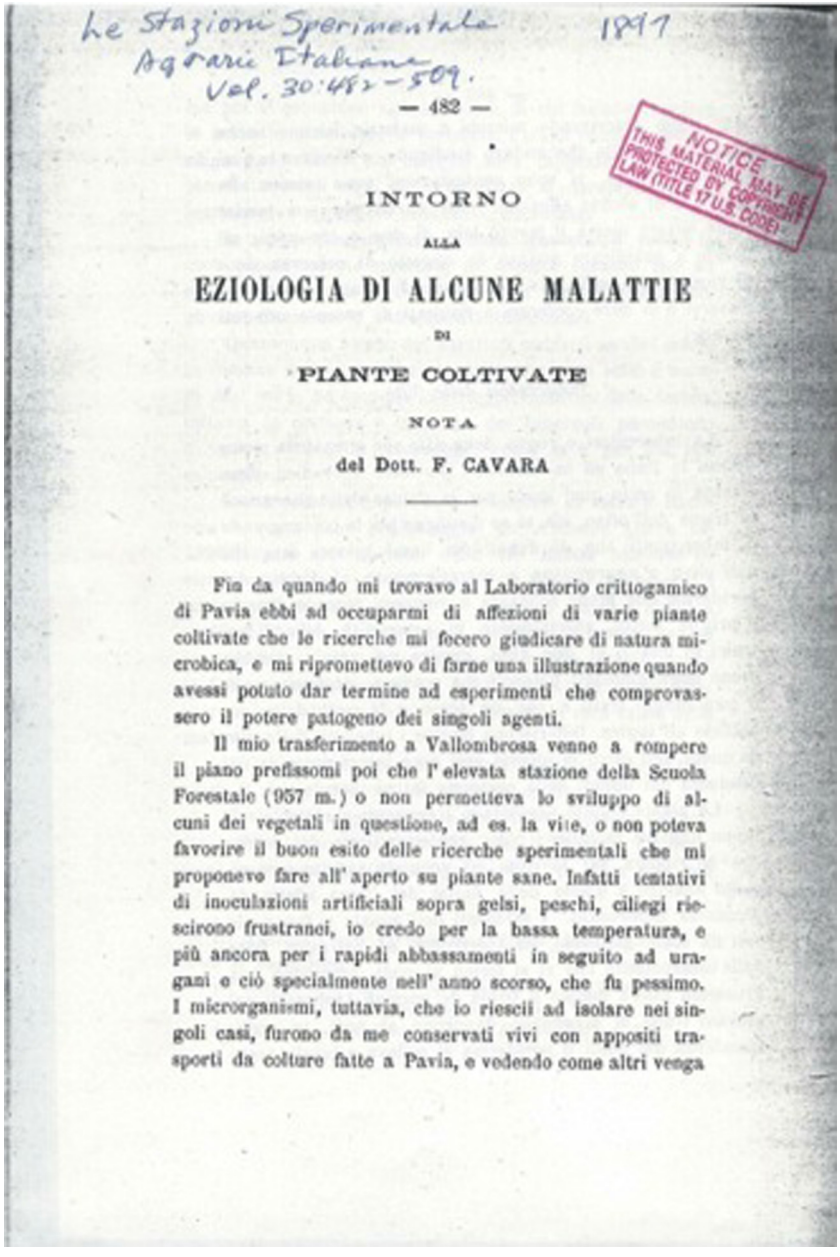
Crown gall is a name given to abnormal tumor-like growths often observed at the base of the trunk and roots of trees, grapevines, and woody plants. The nature of the cause of crown gall was unknown before 1897. Not referenced by many authors who worked on this disease was the published work of Fridiano Cavara (**Figure 1**). He described in detail the galls formed at the base of grapevines that were in the Royal Botanical Gardens of Napoli (Naples), Italy. More importantly, he also described the isolation of a bacterium that he showed caused similar tumors on young grapevines. This work was published in Le Stazioni Sperimentale, Agrari Italiane (Cavara, 1897a,b; **Figure 2**). In 1904, George C. Hedgcock reported the isolation of a causal bacterium from grapevine galls that he described in a US Department of Agriculture Bureau of Plant Industry bulletin (Hedgcock, 1910, p. 21; **Figure 3**). His monograph remains not frequently cited. Most cited as allegedly the first to isolate the causal bacterium was Smith and Townsend (1907). The authors named the causal organism *Bacterium tumefaciens*. E. F. Smith had visited Cavara in Naples and learned how to isolate the causal bacterium from grapevine galls (Rodgers, 1952). He and C. O. Townsend then published the isolation of the crown gall causing bacterium from chrysanthemum. Smith worked extensively on the disease and showed that *B. tumefaciens* can induce gall formation in a number of herbaceous plants (Smith, 1911b). Subsequently, the name *B. tumefaciens* was changed briefly to *Pseudomonas tumefaciens* (Duggar, 1909) and then to *Phytomonas tumefaciens* (Bergey et al., 1923), followed by *Polymonas tumefaciens* (Lieske, 1928), and to *Agrobacterium tumefaciens* (Conn, 1942). The varying phases of the life cycle of *P. tumefaciens* were described by Stapp and Bortels (1931).

In France, Fabre and Dunal (1853) named the tumors observed on diseased grapevines as "broussin." Dornfield (1859) called the galls found on grapevines in Germany as "Grind," but the gall disease was also called "Ausschlag," "Mauche," "Krebs," "Kropf," "Raude," and "Schorf." In Italy, the gall disease on grapevines was called "rogna" (Garovaglio and Cattaneo, 1879) and "tubercoli" (Cavara, 1897a,b). In the United States, the gall disease observed on grapevines was called "black-knot" (Galloway, 1889) and likewise in Canada (Fletcher, 1890). Other names such as tubercular galls were applied to this tumorous disease that had become recognized throughout the continents wherever grapevines and woody crops were cultivated.

Eventually, nurserymen, farmers, viticulturalists, etc., became aware of the gall producing disease that occurred at the base of trees and vines near the junction of the roots to the trunk, known to these growers as the "crown," the term "crown-gall" became the common name used to recognize the tumor-forming disease.

SEARCH FOR THE AGENT THAT CAUSED CROWN GALL

Once *A. tumefaciens* was established as the cause of crown gall, the quest was initiated for the mechanism by which this pathogen induced tumors in plants. It was widely known that *A. tumefaciens* induces tumors readily by mechanical inoculation of many different plant species. Eventually, over 90 families of plants were found to be susceptible to Crown Gall disease incited by this



Le Stazioni Sperimentale Agrarie Italiane Vol. 30: 482–509.

1897

INTORNO

ALLA

EZIOLOGIA DI ALCUNE MALATTIE

DI

PIANTE COLTIVATE

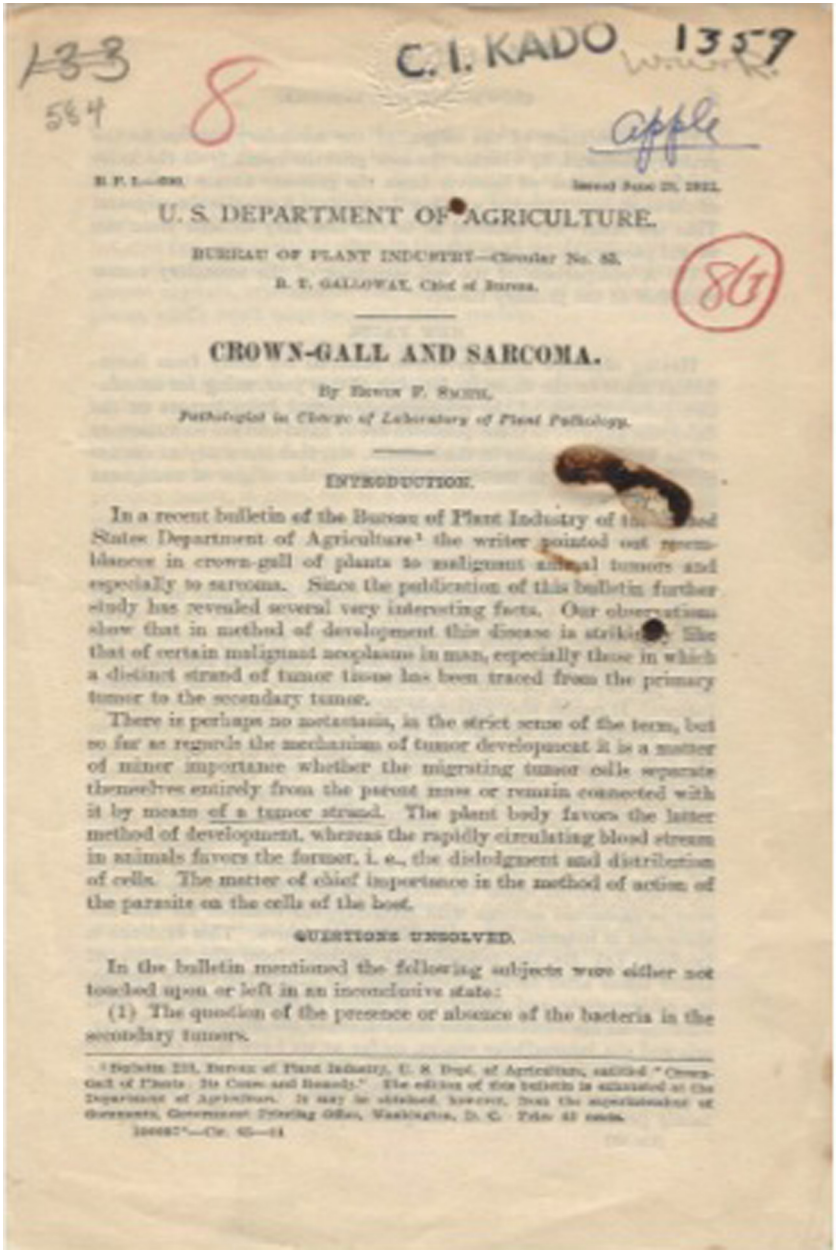
NOTA

del Dott. F. CAVARA

Fin da quando mi trovavo al Laboratorio crittogamico di Pavia ebbi ad occuparmi di affezioni di varie piante coltivate che le ricerche mi fecero giudicare di natura microbica, e mi ripromettevo di farne una illustrazione quando avessi potuto dar termine ad esperimenti che comprovassero il potere patogeno dei singoli agenti.

Il mio trasferimento a Vallombrosa venne a rompere il piano prefissomi poi che l'elevata stazione della Scuola Forestale (957 m.) o non permetteva lo sviluppo di alcuni dei vegetali in questione, ad es. la vite, o non poteva favorire il buon esito delle ricerche sperimentali che mi proponevo fare all'aperto su piante sane. Infatti tentativi di inoculazioni artificiali sopra gelsi, peschi, ciliegi riescirono frustranei, io credo per la bassa temperatura, e più ancora per i rapidi abbassamenti in seguito ad uragani e ciò specialmente nell'anno scorso, che fu pessimo. I microrganismi, tuttavia, che io riescii ad isolare nei singoli casi, furono da me conservati vivi con appositi trasporti da colture fatte a Pavia, e vedendo come altri venga

FIGURE 2 | Paper by Fridiano Cavara in 1897 describing galls on grapevines from which he isolated the tumorigenic bacterium and demonstrated its gall forming activity on young grapevines.



B. P. I.—680. Issued June 20, 1911.

U. S. DEPARTMENT OF AGRICULTURE.

BUREAU OF PLANT INDUSTRY—Circular No. 85.

B. T. GALLOWAY, Chief of Bureau.

CROWN-GALL AND SARCOMA.

By ERWIN F. SMITH,

Pathologist in Charge of Laboratory of Plant Pathology.

INTRODUCTION.

In a recent bulletin of the Bureau of Plant Industry of the United States Department of Agriculture[1] the writer pointed out resemblances in crown-gall of plants to malignant animal tumors and especially to sarcoma. Since the publication of this bulletin further study has revealed several very interesting facts. Our observations show that in method of development this disease is strikingly like that of certain malignant neoplasms in man, especially those in which a distinct strand of tumor tissue has been traced from the primary tumor to the secondary tumor.

There is perhaps no metastasis, in the strict sense of the term, but so far as regards the mechanism of tumor development it is a matter of minor importance whether the migrating tumor cells separate themselves entirely from the parent mass or remain connected with it by means of a tumor strand. The plant body favors the latter method of development, whereas the rapidly circulating blood stream in animals favors the former, i. e., the dislodgment and distribution of cells. The matter of chief importance is the method of action of the parasite on the cells of the host.

QUESTIONS UNSOLVED.

In the bulletin mentioned the following subjects were either not touched upon or left in an inconclusive state:

(1) The question of the presence or absence of the bacteria in the secondary tumors.

[1] Bulletin 231, Bureau of Plant Industry, U. S. Dept. of Agriculture, entitled "Crown-Gall of Plants: Its Cause and Remedy." The edition of this bulletin is exhausted at the Department of Agriculture. It may be obtained, however, from the superintendent of Documents, Government Printing Office, Washington, D. C. Price 45 cents.

100687°—Cir. 85—11

FIGURE 4 | Paper published in 1911 by Erwin F. Smith describing the similarities and differences between crown gall and human sarcoma.

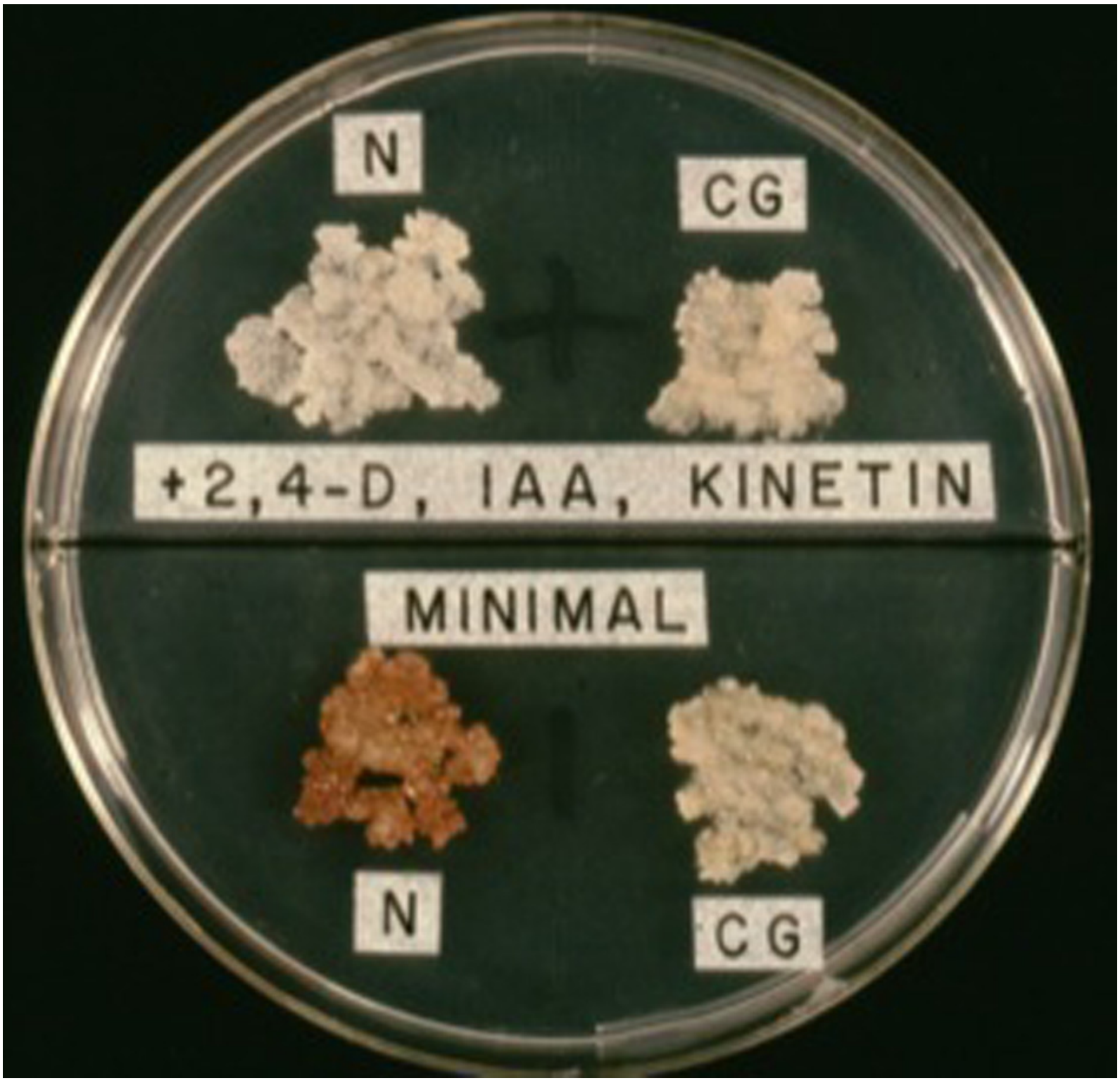


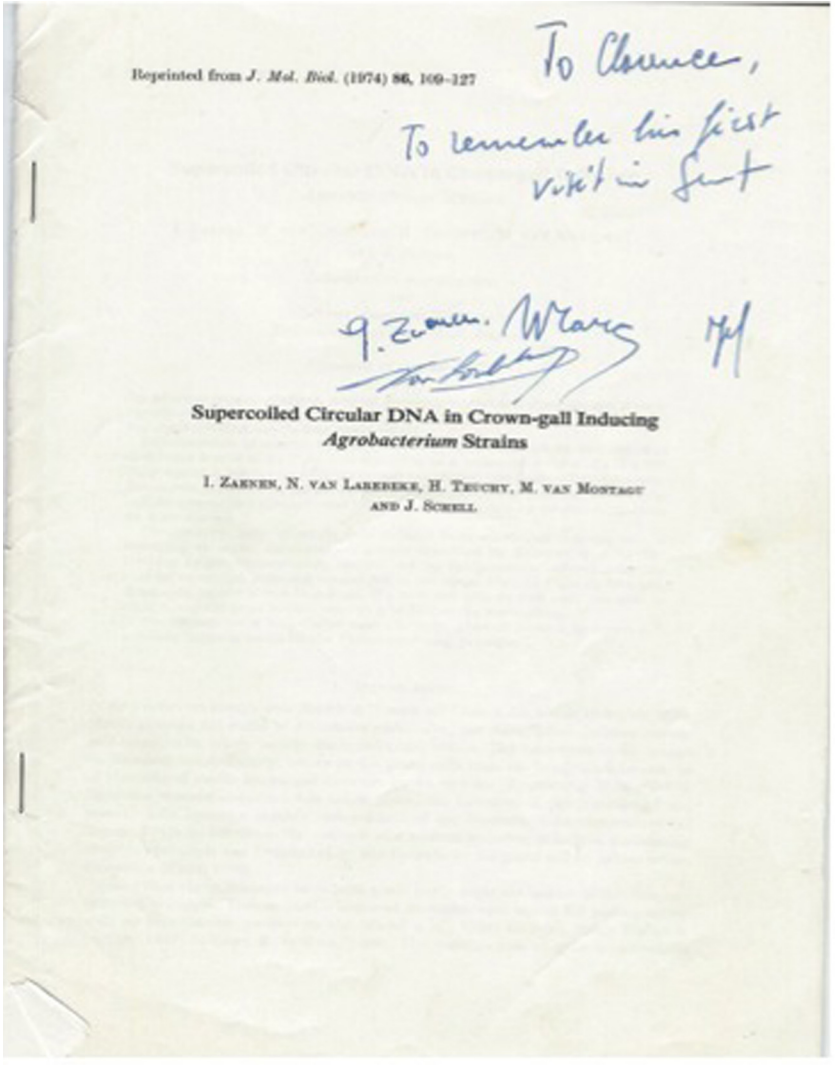
FIGURE 7 | Auxin autotrophy of crown gall tissues on hormone-free medium (lower half of bisectored petri plate). N, normal cells; CG, crown gall cells; 2,4-D, 2,4-phenoxyacetic acid; IAA, indole-3-acetic acid.

Tumor-causing ability (virulence) of Agrobacterium correlates with the presence of a large extrachromosomal element in the bacterium - the Ti plasmid

- All virulent bacteria have this plasmid
- Curing virulent strains of the plasmid eliminates virulence
- Transposon disruptions in the Ti plasmid alter or eliminate virulence

Transposons can be used to generate mutants, create a genetic map of the Ti plasmid

- Mobilize transposon (natural or tailored) from E. coli into Agrobacterium using a plasmid that replicates in E. coli but not Agrobacterium (takes advantage of the ability of episome or plasmid transfer systems to move plasmids between different species of gram-negative bacteria)
- Select for Agrobacteria that retain the selectable marker carried by the transposon
- Screen for virulence
- Test for chromosomal vs. extrachromosomal linkage



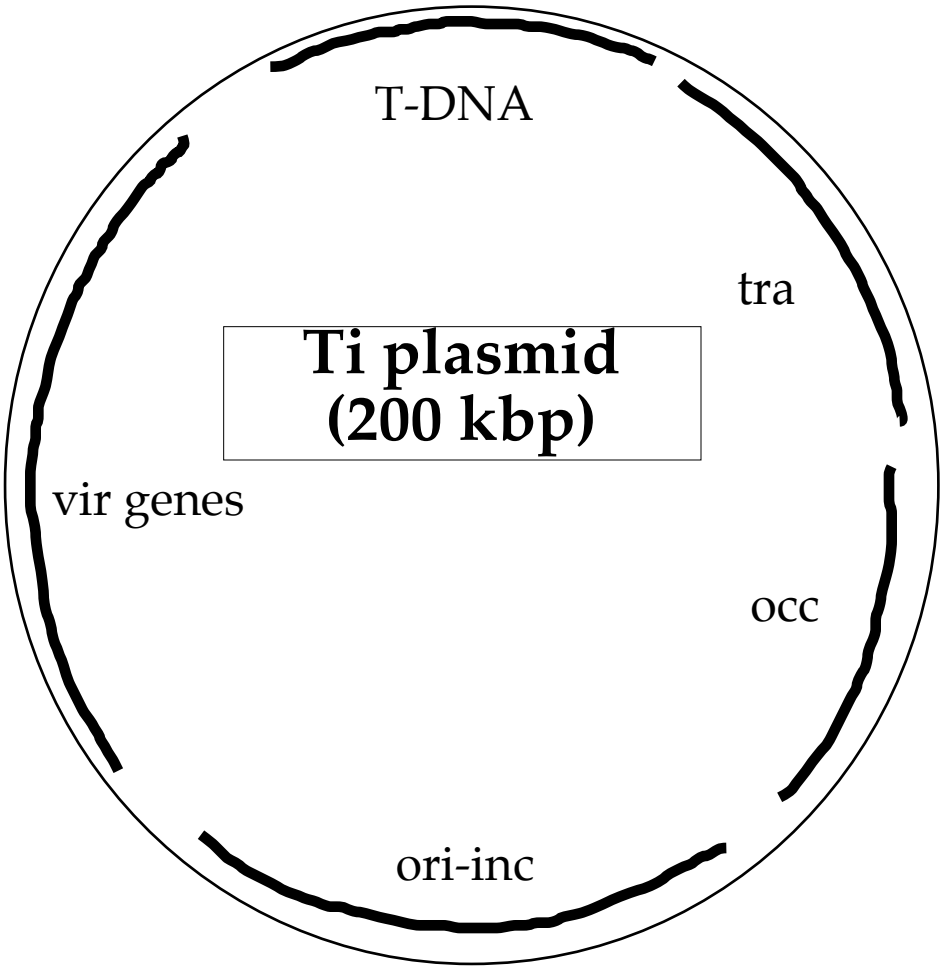
Reprinted from *J. Mol. Biol.* (1974) **86**, 109–127

To Clarence,
To remember his first
visit in Gent

Supercoiled Circular DNA in Crown-gall Inducing *Agrobacterium* Strains

I. Zaenen, N. van Larebeke, H. Teuchy, M. van Montagu and J. Schell

FIGURE 9 | Classic paper first reporting the presence of an *A. tumefaciens* plasmid associated with tumorigenicity by Jeff Schell's laboratory.


T-DNA
tra
Ti plasmid
(200 kbp)
vir genes
occ
ori-inc

T-DNAs of Ti plasmids

Transposon mutations in the T-DNA region change tumor morphology or abolish tumor growth

T-DNA-related sequences can be found in transformed plant cells that are free of Agrobacterium

T-DNA-related RNAs (that are polyadenylated) can be detected in axenically-grown tumors

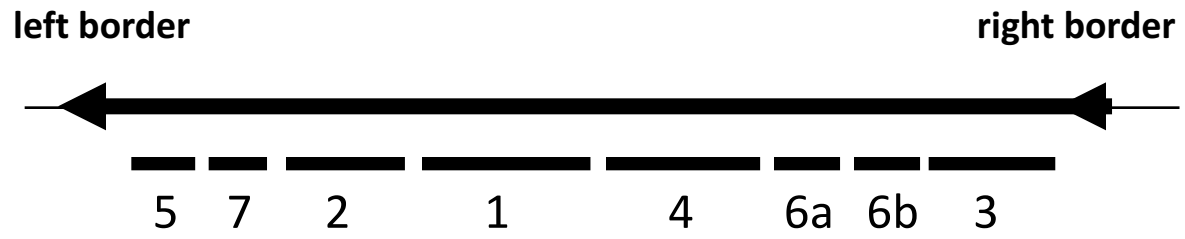
-> Transformed plant cells contain a part of the Ti plasmid (but not the entire plasmid)

the T-DNA contains eight potential genes - these are eukaryotic in nature (eukaryotic promoters, monocistronic, eukaryotic polyadenylation signals, eukaryotic translation mechanisms)

several T-DNA genes encode enzymes that can enable cytokinin and auxin production

-> crown gall tumorigenesis is due to the "activation" of unregulated phytohormone synthesis in the transformed cells

octopine TL-DNA



arginine + pyruvate —(3)→ **octopine**

tryptophan —(1)→ **indoleacetamide** —(2)→ **indoleacetic acid (auxin)**

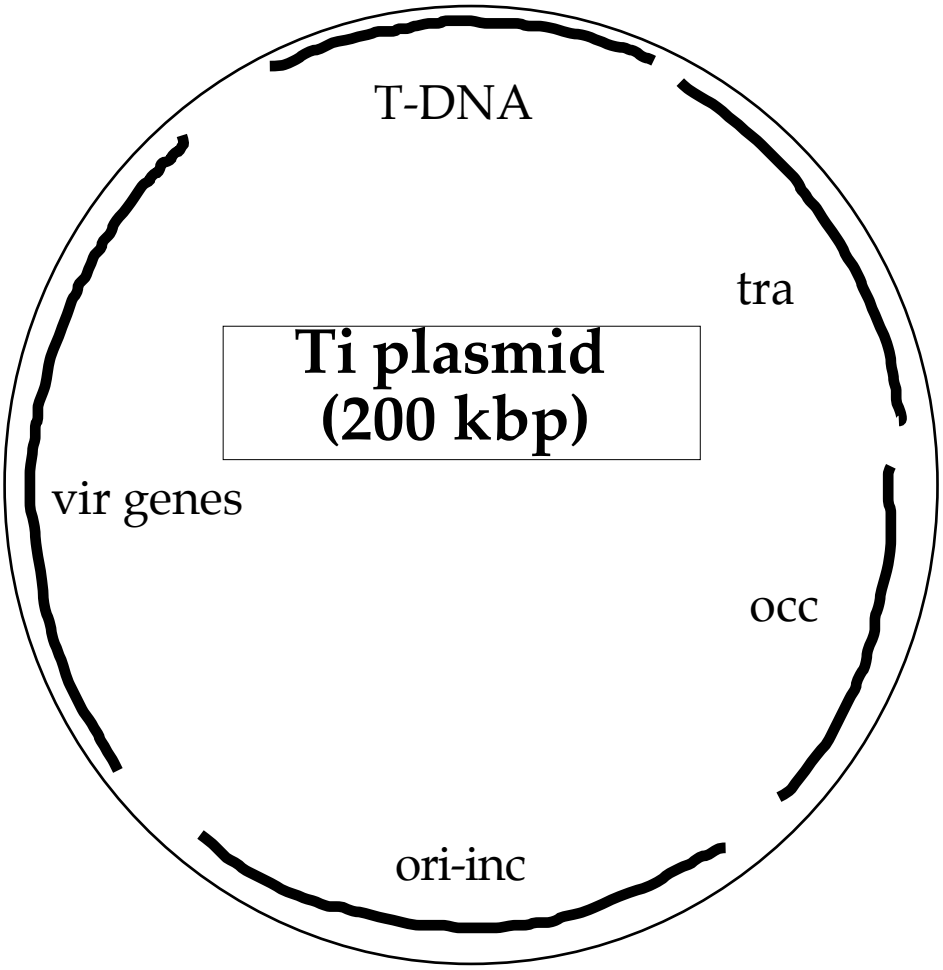
AMP + dimethyl allylpyrophosphate —(4)→ **cytokinin**

tryptophan —(5)→ **indole-3-lactate (an auxin antagonist)**

6b **promotes tumorogenic growth (transcriptional controls?)**

6a **octopine secretion**

7 **unknown function (if any)**


T-DNA
tra
Ti plasmid
(200 kbp)
vir genes
occ
ori-inc

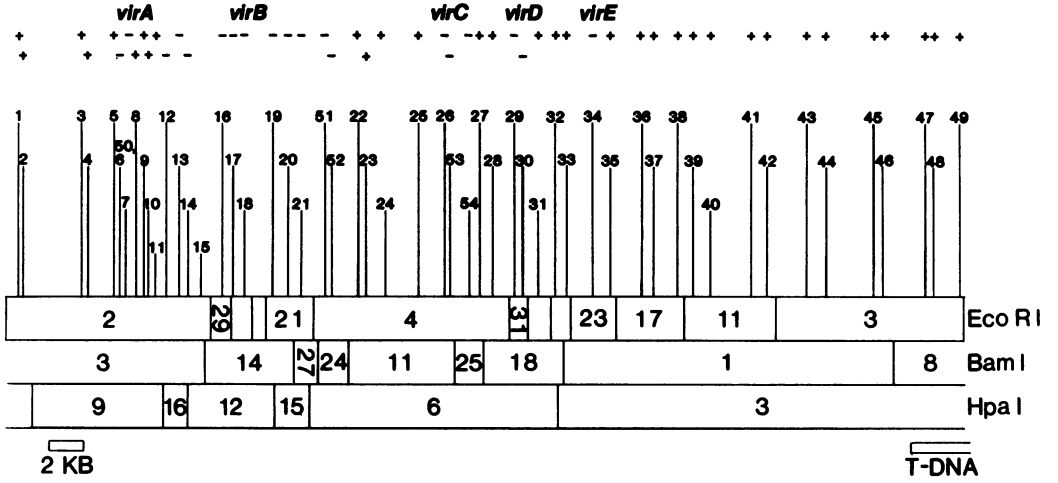
The vir region of Ti plasmids

Not found in transformed plant cells

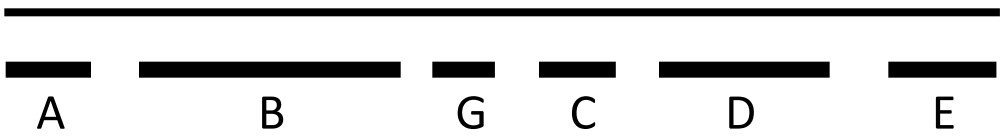
Transposon mutations in the vir region usually abolish tumor growth

Genetic analysis -> several genetic complementation groups

Sequence and characterize …..


virA
virB
virC
virD
virE
Eco R I
Bam I
Hpa I
2 KB
T-DNA

the vir region of Ti plasmids



vir locus	function
A	**Regulatory (recognizes plant metabolites, activates virG)**
G	**Regulatory (transcriptional activator of other vir loci)**
D	**Nicks Ti plasmid at T-DNA borders, covalently attaches to "T-strand"**
C	**Recognizes "overdrive" sequences near right border of T-DNA, increases T-strand production**
E	**ss-DNA binding protein (mediates T-strand production and transit in plant cells)**
B	**Transfer apparatus**

Regulation of vir gene expression

Most vir genes are inducible, vir genes can be induced by exposure of Agrobacterium to exudates isolated from wounded plant cells

Many chemicals can induce vir gene expression; these are typified by phenolic compounds such as acetosyringone

Two vir loci are single genes and are expressed constitutively

Mutations in these (virA and virG) are pleiotropic - they affect expression of all other vir genes

VirA encodes a membrane-localized protein that resembles components of so-called two component sensor-regulator systems

The virA gene product can phosphorylate itself on a characteristic histidine residue, and can transfer this phosphate to an aspartate side chain of the virG gene product

VirG encodes a DNA binding protein that is an acceptor of phosphate from phosphorylated virA

The virG gene product can bind elements present in other vir gene promoters

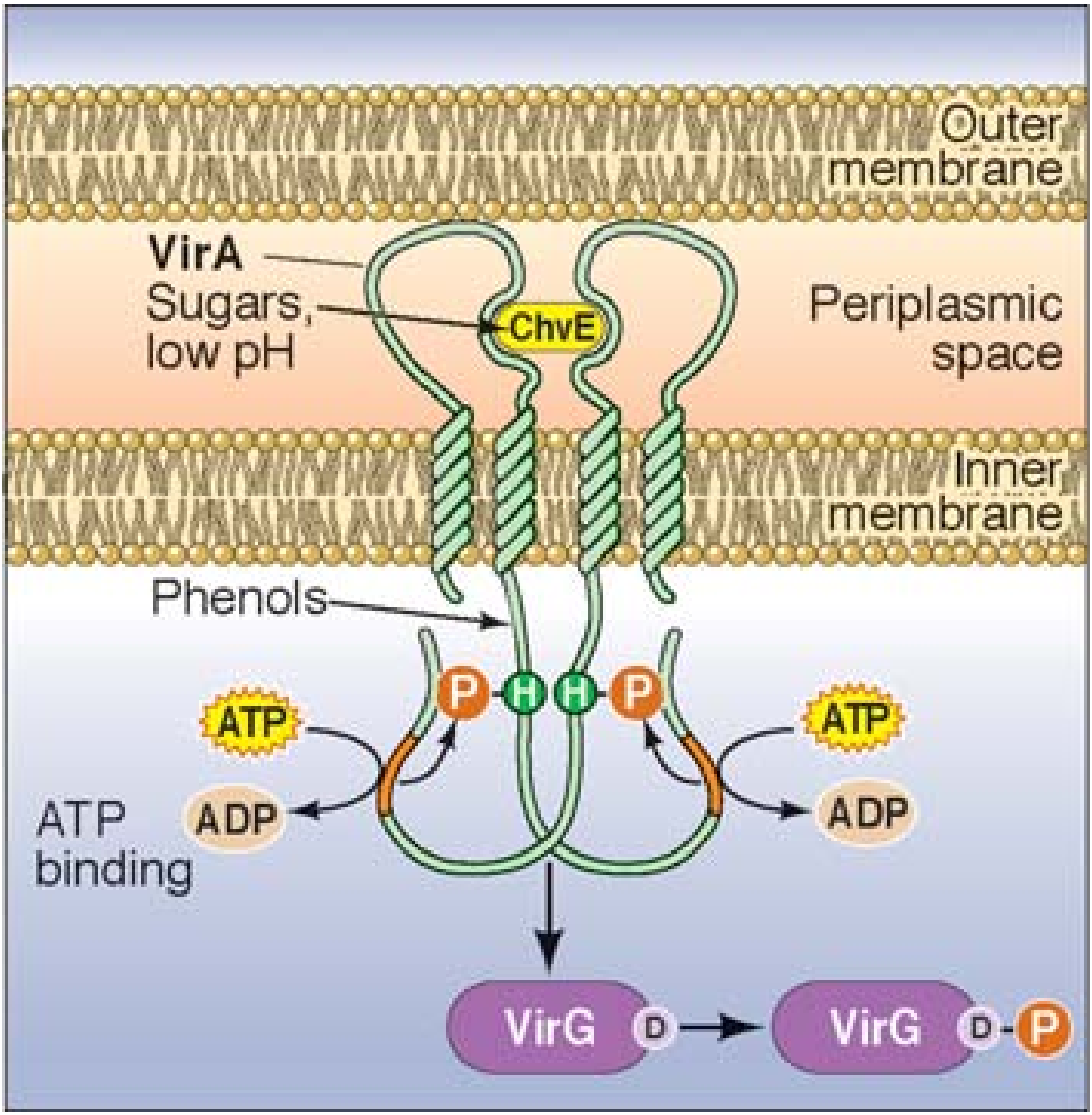


Figure 2

The ChvE/VirA/VirG signal transducing system (note that stoichiometry of ChvE:VirA is not known). See text for details.

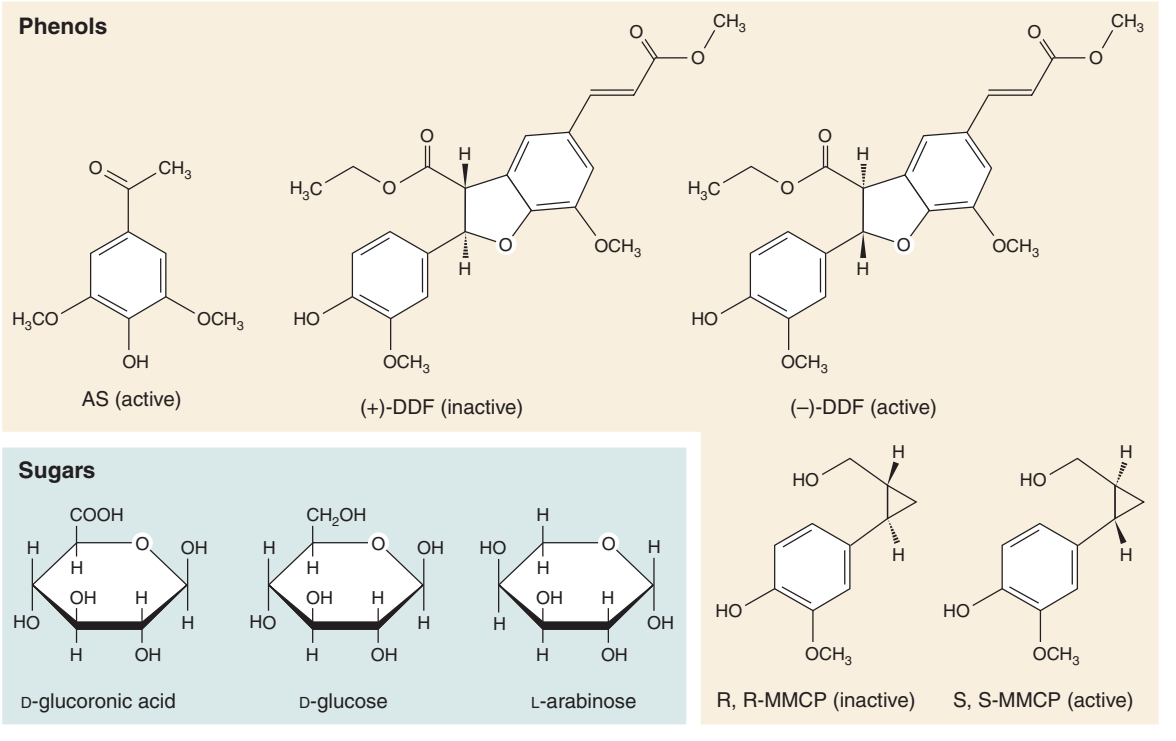


Figure 3

Representative phenols and sugars (pyranose forms) capable of serving as signals to induce *vir* gene expression. Active versus inactive enantiomers of DDF and MMCP are shown. AS, acetosyringone; DDF, dehydrodiferulate dimethylester; MMCP, 1-hydroxymethyl-2-(4-hydroxy-3-methoxyphenyl)-cyclopropane.

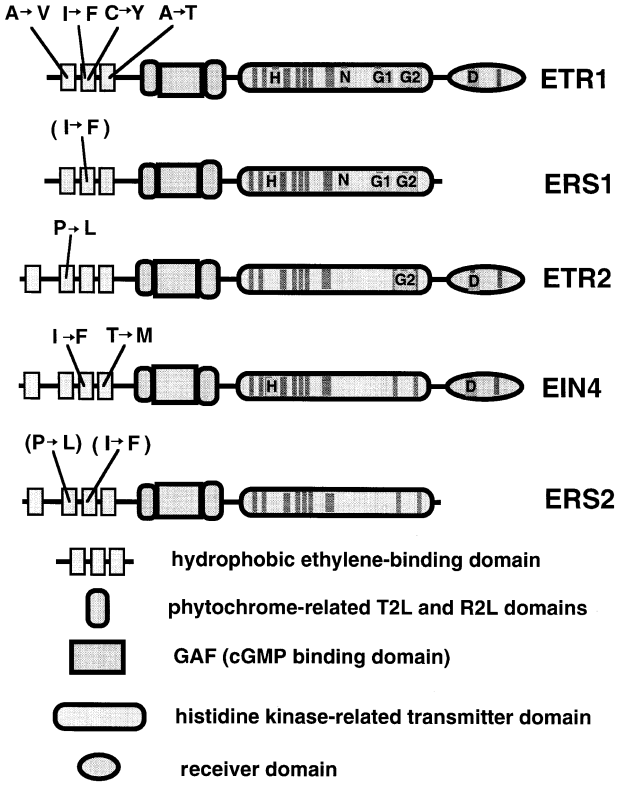
“Two-component” signaling and regulatory systems

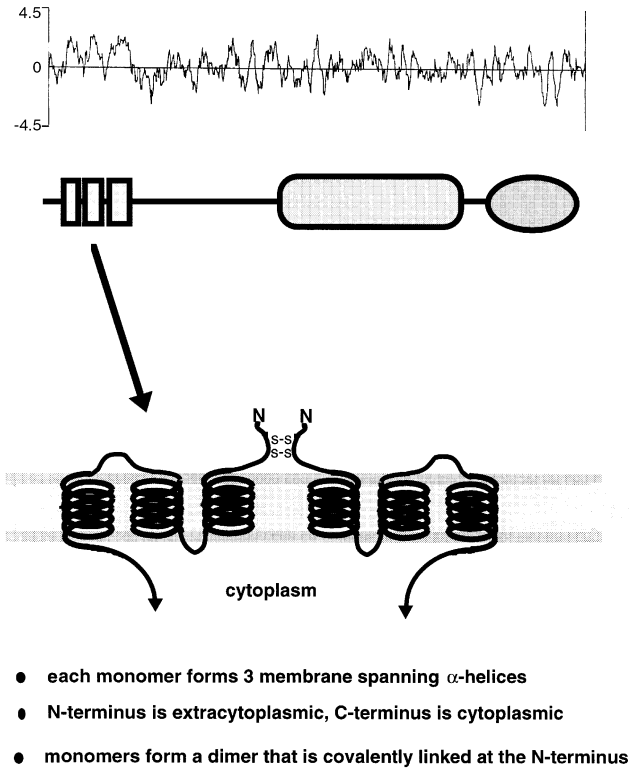
A common mechanism for regulation of gene expression in bacteria

Also seen in plants, a mechanism for regulation by ethylene and cytokinins

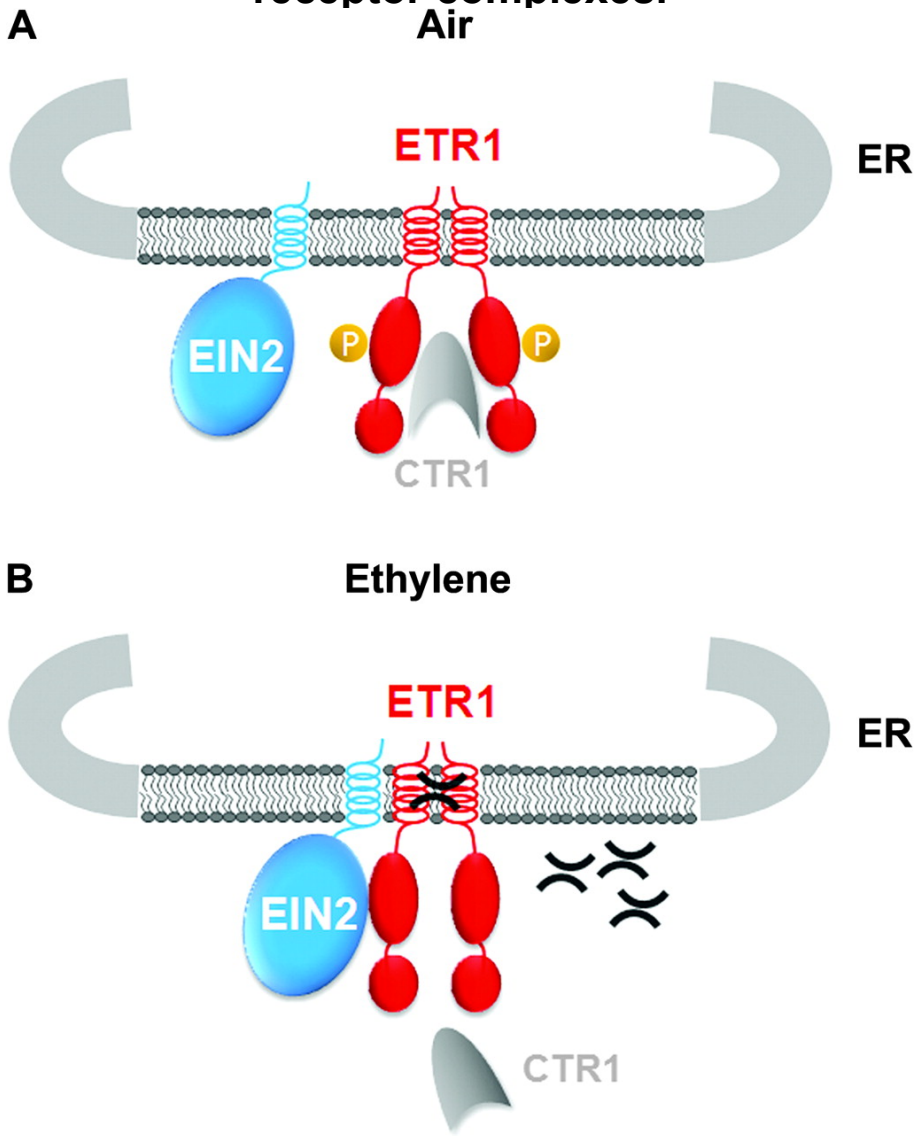
Examples of systems in bacteria that are regulated by two-component systems:

- **Sensing of oxygen and redox states**
- **Nitrate and nitrite respiration**
- **Transport and anaerobic metabolism of citrate**
- **Chemotaxis**
- **Nitrogen fixation**
- **Bacterial cell attachment**
- **Degradation of benzene derivatives**
- **Nitrogen utilization**
- **K+ supply**
- **Antibiotics**
- **Osmolarity**
- **Sporulation**
- **Virulence**
- **Quorum sensing**
- **Lipid modification**


A→V I→F C→Y A→T
H N G1 G2 D
ETR1
(I→F)
H N G1 G2
ERS1
P→L
G2 D
ETR2
I→F T→M
H D
EIN4
(P→L) (I→F)
ERS2
hydrophobic ethylene-binding domain
phytochrome-related T2L and R2L domains
GAF (cGMP binding domain)
histidine kinase-related transmitter domain
receiver domain


4.5
0
-4.5
N N
S-S
S-S
cytoplasm
each monomer forms 3 membrane spanning α-helices
N-terminus is extracytoplasmic, C-terminus is cytoplasmic
monomers form a dimer that is covalently linked at the N-terminus

Signaling Complexes Formed at the ER Membrane in Response to Ethylene.Schematic model of the processes induced at the ER membrane by the binding of the plant hormone to the ethylene receptor complexes.



Bisson M M , Groth G Mol. Plant 2010;3:882-889

MOLECULAR PLANT

Agrobacterium tumefaciens and Plant Cell Interactions and Activities Required for Interkingdom Macromolecular Transfer

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1081-0706/06/1110-0101$20.00

Key Words

virA-virG two-component system, signal integration, type IV secretion, VirB complex

Abstract

Host recognition and macromolecular transfer of virulence-mediating effectors represent critical steps in the successful transformation of plant cells by *Agrobacterium tumefaciens*. This review focuses on bacterial and plant-encoded components that interact to mediate these two processes. First, we examine the means by which *Agrobacterium* recognizes the host, via both diffusible plant-derived chemicals and cell-cell contact, with emphasis on the mechanisms by which multiple host signals are recognized and activate the virulence process. Second, we characterize the recognition and transfer of protein and protein-DNA complexes through the bacterial and plant cell membrane and wall barriers, emphasizing the central role of a type IV secretion system—the VirB complex—in this process.

THE ROYAL SOCIETY

The ethylene-receptor family from *Arabidopsis*: structure and function

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The gaseous hormone ethylene regulates many aspects of plant growth and development. Ethylene is perceived by a family of high-affinity receptors typified by the ETR1 protein from *Arabidopsis*. The *ETR1* gene codes for a protein which contains a hydrophobic N-terminal domain that binds ethylene and a C-terminal domain that is related in sequence to histidine kinase–response regulator two-component signal transducers found in bacteria. A structural model for the ethylene-binding domain is presented in which a Cu(I) ion is coordinated within membrane-spanning α-helices of the hydrophobic domain. It is proposed that binding of ethylene to the transition metal would induce a conformational change in the sensor domain that would be propagated to the cytoplasmic transmitter domain of the protein. A total of four additional genes that are related in sequence to *ETR1* have been identified in *Arabidopsis*. Specific missense mutations in any one of the five genes leads to ethylene insensitivity *in planta*. Models for signal transduction that can account for the genetic dominance of these mutations are discussed.

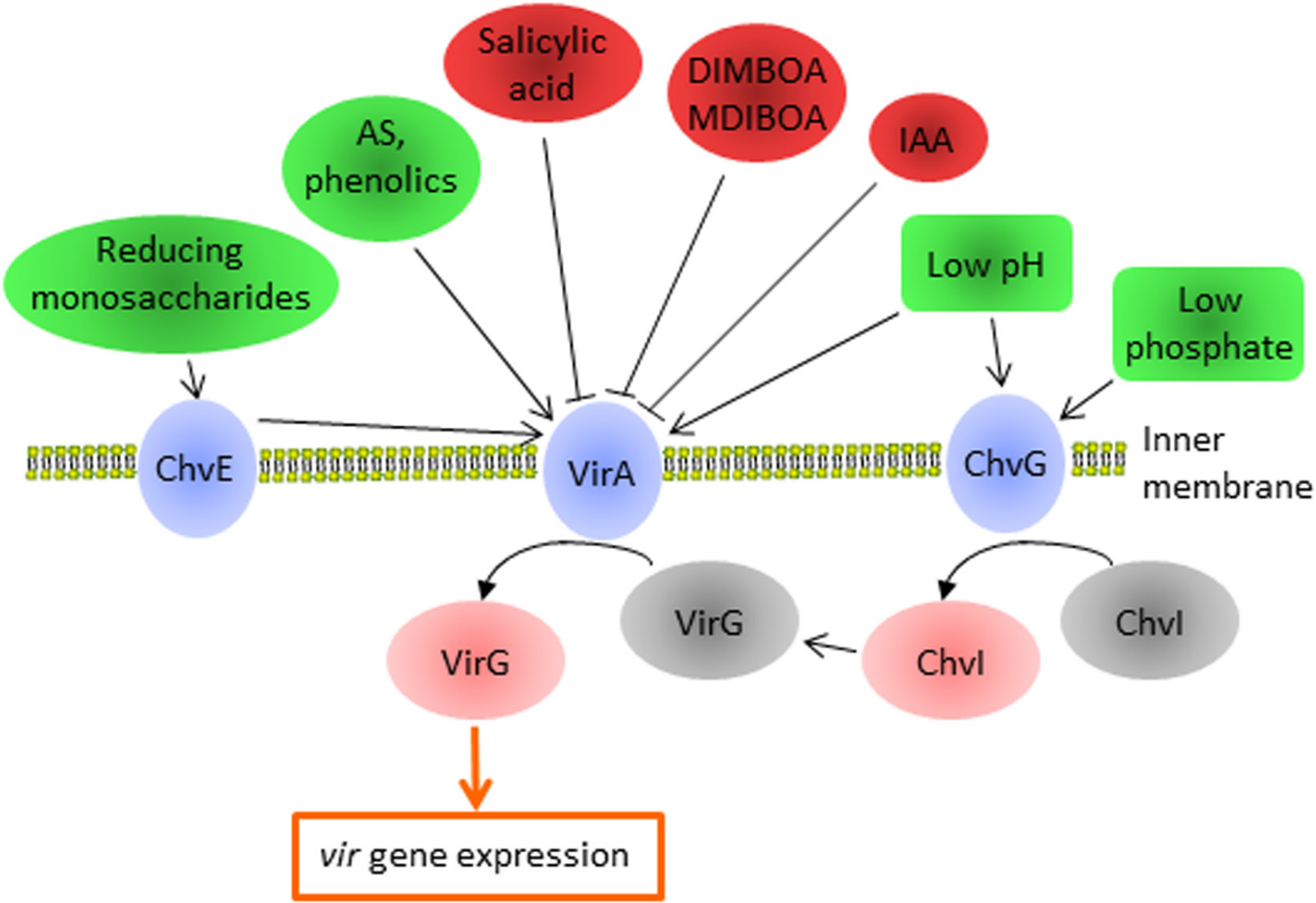
Keywords: ethylene; receptor; signal transduction; plant hormone

1. INTRODUCTION

While the concept of small gas molecules acting as biological signals may be something of a novelty in animal systems, almost a century has passed since Neljubov (Abeles *et al.* 1992) demonstrated that nanomolar concentrations of ethylene could elicit dramatic effects on plant growth and development. In addition to the commonly recognized role in fruit ripening, ethylene influences a range of developmental processes throughout the life cycles of higher plants. The stimulation of seed germination, the adjustment of seedling growth to varying soil conditions, the rate and extent of leaf expansion, and the timing of vegetative senescence and abscision are just a few of the plant processes that are regulated by ethylene. Ethylene may also mediate responses to environmental challenges such as wounding, pathogen invasion, and water stress (Abeles *et al.* 1992; Ecker 1995; Bleecker & Schaller 1996; Keiber 1997).

Despite a century of scientific investigation, the mechanisms by which this small gas molecule works at such low concentrations to influence so many different processes in plants remained a mystery. Burg & Burg were the first to hypothesize that ethylene might interact with a transition metal cofactor coordinated in the presumptive receptor for ethylene (Burg & Burg 1965, 1967). In the 1970s and 1980s, investigators in the USA (Sisler 1979) and the UK (Sanders *et al.* 1989, 1991) identified and characterized saturable ethylene binding sites in a variety of plant tissues using ^{14}C-labelled ethylene. While these sites showed sufficiently high affinity for ethylene to account for biological activity, no direct connection could be made between this ethylene binding and *in vivo* responses to ethylene. Early events in the transduction of the ethylene signal were also a complete mystery. The study of these processes has come of age in the past decade through the use of mutational analysis in *Arabidopsis* to identify the genes coding for early components in ethylene signal transduction (Bleecker *et al.* 1988; Guzman & Ecker 1990; Chang *et al.* 1993; Kieber *et al.* 1993; Roman *et al.* 1995).

The genetic approach to identify the biochemical components involved in ethylene signal transduction is a deceptively simple one, and it illustrates the power of the method. Ethylene inhibits the elongation growth of etiolated (dark-grown) seedlings. Ethylene-insensitive mutants were readily identified in dark-grown seedlings incubated in ethylene (Bleecker *et al.* 1988; Guzman & Ecker 1990; Kieber *et al.* 1993; Roman *et al.* 1995; Chao *et al.* 1997). Insensitive mutants obtained in these screens have been extensively characterized and many of the represented genes have been cloned. This pioneering work by a number of research groups has been the subject of a number of recent reviews (Ecker 1995; Bleecker & Schaller 1996; Kieber 1997; Fluhr 1998) and will not be covered in detail here. Briefly, the framework of the early signal transduction pathway can be proposed from the characteristics of the identified genes. The perception of ethylene is apparently achieved by catalytic receptors coded for by the *ETR1*-like gene family (Chang *et al.* 1993; Hua *et al.* 1995, 1998; Sakai *et al.* 1998). ETR1 is related in structure and sequence to the two-component regulators from bacteria which transduce signals via phosphotransfer reactions (Parkinson 1993). This receptor system signals downstream via an RAF kinase-like negative regulator, designated CTR1,


Salicylic acid
DIMBOA MDIBOA
AS, phenolics
IAA
Reducing monosaccharides
Low pH
Low phosphate
ChvE
VirA
ChvG
Inner membrane
VirG
VirG
ChvI
ChvI
vir gene expression

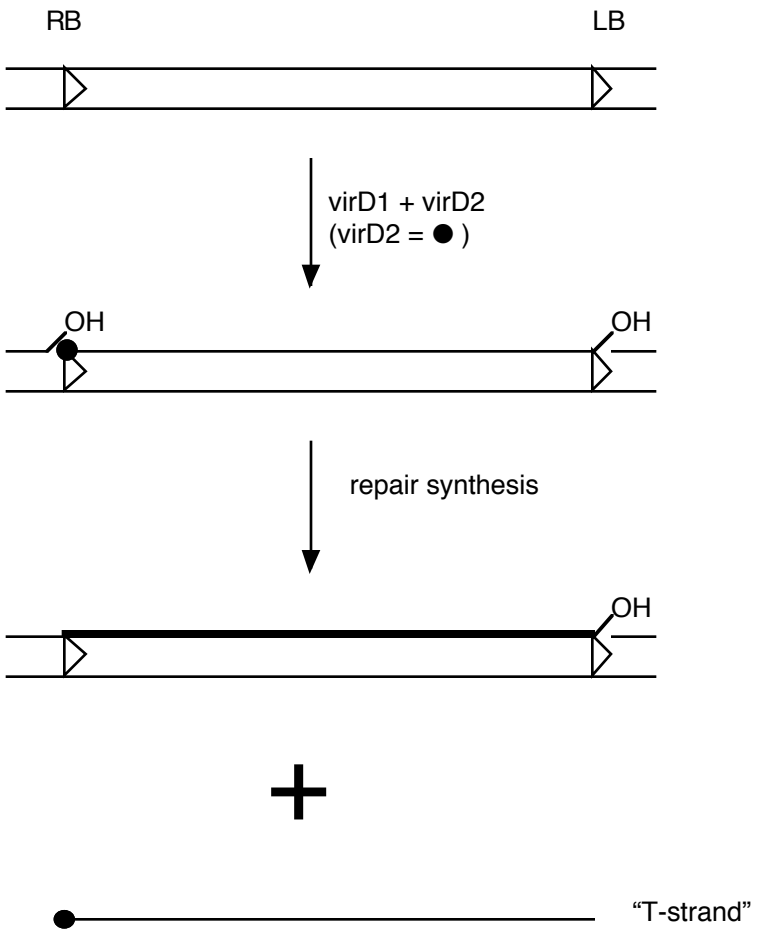
forms of T-DNA that are found in Agrobacterium

- ds circles - found only in induced bacteria, not (apparently) in plant cells
- ds linear T-DNA - found only in induced bacteria, not (apparently) in plant cells
- ss linear T-DNA - found in bacteria and plant cells
- what is not found - Ti plasmids with evidence that T-DNA has been precisely deleted

In vitro activities of virD proteins

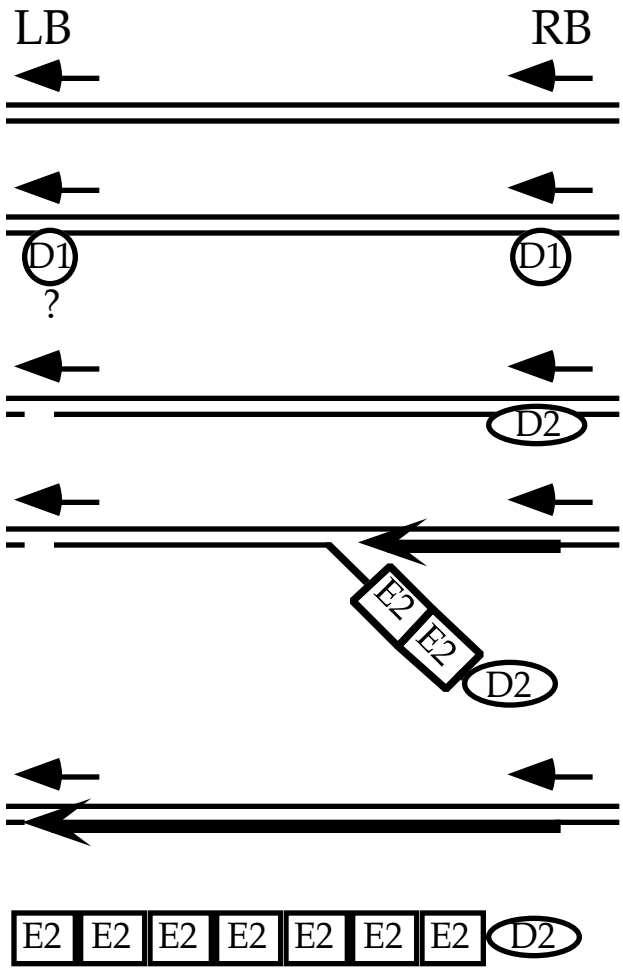
- virD1 + virD2 - recognize T-DNA borders with high specificity, introduce single-stranded nicks at borders
- virD2 alone can work on oligonucleotides containing the border
- virD1 as well is required for action on supercoiled circular DNA
- no reaction is seen with relaxed circles or linear ds molecules

production of “T-strands” in Agrobacterium



virE2 - where and how does it work?

- ss DNA binding protein
- can be found associated with T-strands in Agrobacterium extracts (co-immunoprecipitation)
- wt levels of T-strands accumulate in virE2 mutants (virE2 not needed for T-strand production or in vivo stabilization)
- virE2 mutants can transfer T-strands to plant cells (efficiency is lower) - no absolute requirement for transfer
- virE2 mutants can be complemented by expression of virE2 in plant cells - is the main site of virE2 function in the plant?
- virE2(+), T-DNA (-) + virE2(-), T-DNA (+) combination is virulent and tumorigenic - can virE2 be transferred from Agrobacterium to plant cell independent of T-DNA?


LB
RB
D1
D1
?
D2
E2
E2
D2
E2
E2
E2
E2
E2
E2
E2
D2

Similarities between virB gene products and the transfer apparatus of F plasmids

Conjugation	function, location	Ti plasmid
Tra L	transglycosylase, E	VirB1
	cell-cell contact, E	VirB1*
TraM	pilin subunit, E	VirB2
TraA	unknown, E	VirB3
TraB	ATPase, transport, TM	VirB4
TraC	unknown, E	VirB5
TraD	pore former?, TM	VirB6
TraN	lipoprotein, covalent dimer with virB9, OM	VirB7
TraE	unknown, PIM	VirB8
TraO	nucleation center with VirB7, OM	VirB9
TraF	unknown,TM	VirB10
TraG	ATPase, transport, IM	VirB11
	ATPase, coupling of DNA processing with transport system, TM	VirD4

Analogies between bacterial conjugation and T-DNA transfer

Conjugation	Function	T-DNA transfer
oriT	site of specific nicking	T-DNA borders
TraI/TraY endonuclease	site-specific endonuclease	VirD1/VirD2
TraD/TraM	DNA transfer (?)	?
?	mating signal	plant-synthesized phenolics
F-pilus (TraA,Q,L,E,K, B,V,C,W,U,F,H,G)	transfer machinery	VirB
TraG/TraN	stabilization of mating pair	?
TraS/TraT	prevention of non-productive mating	chv (?)
ssb	stabilization of T-strands	virE

Conjugation, T-DNA transfer, and secretory systems involved in virulence in animal and human pathogens (*Bordetella pertussis, Legionella pneumophila, Rickettsia prowazekii*) are members of a large class of bacterial secretory systems (type IV systems)

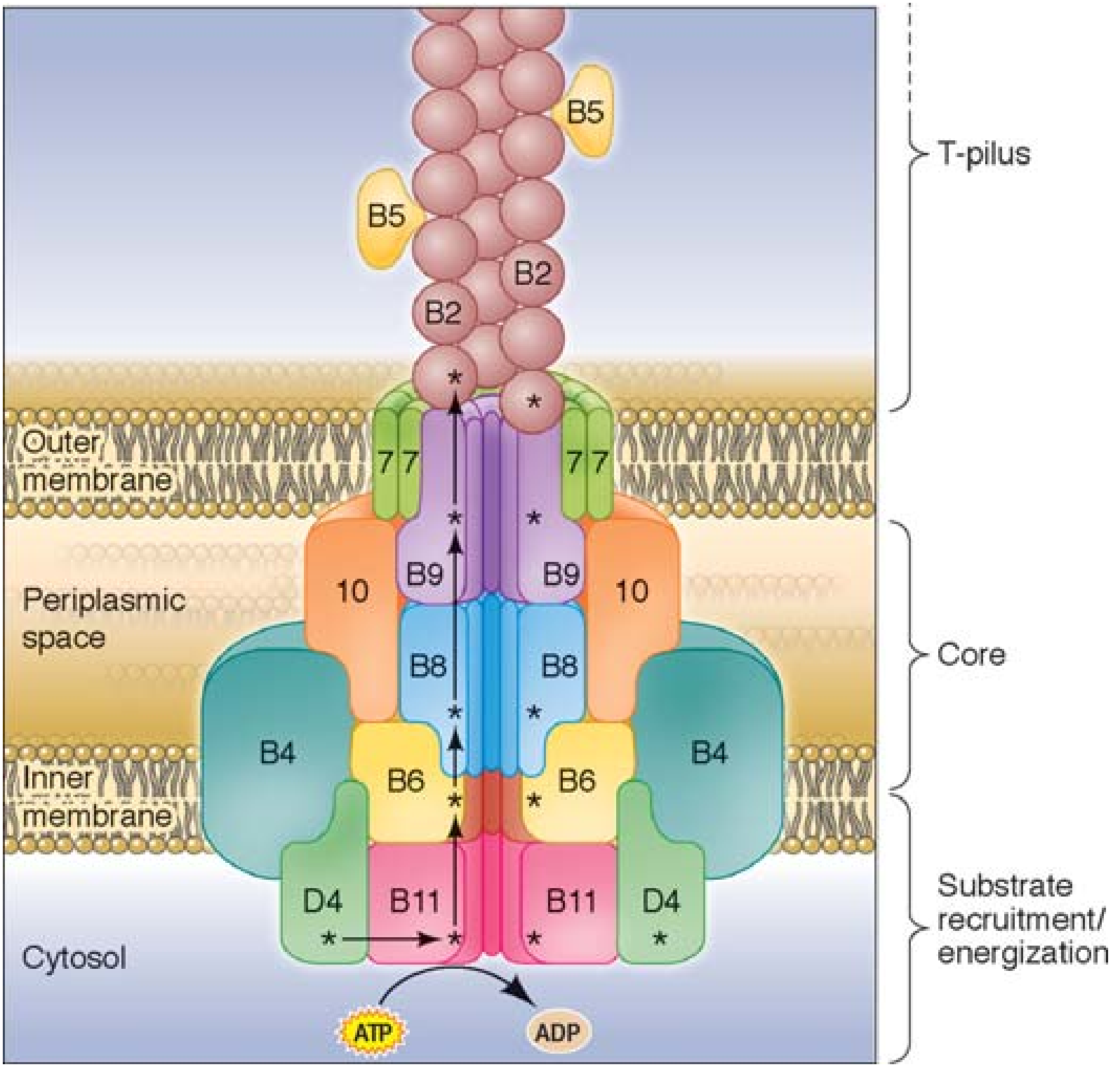


Figure 5

Generalized scheme representing the VirB/D4 complex, depicting localization and some of the known interactions between members of the complex. Asterisks indicate proteins shown to interact with the transported DNA substrate, and arrows indicate the order of DNA transfer through the complex. See text for details.

Beyond the bacterial cell

Once it is made in the bacterial cell, the T-strand has a tortuous route to the nucleus and ultimately the nuclear genome

The various phases in the life of the T-strand involve vir proteins and host proteins

The phases:

transport across the plant cell membrane

movement through the cytoplasm

import into the nucleus

“stripping” of proteins from the T-strand

integration into the nuclear genome

In each of these phases, host proteins are recruited or co-opted to facilitate the respective step

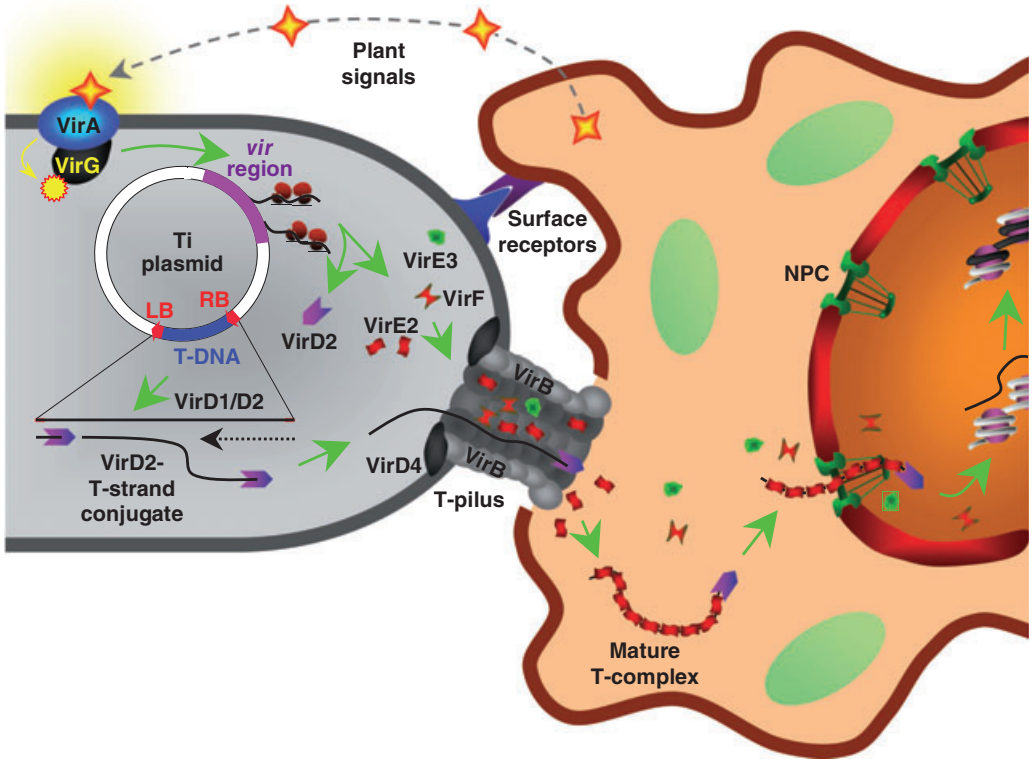
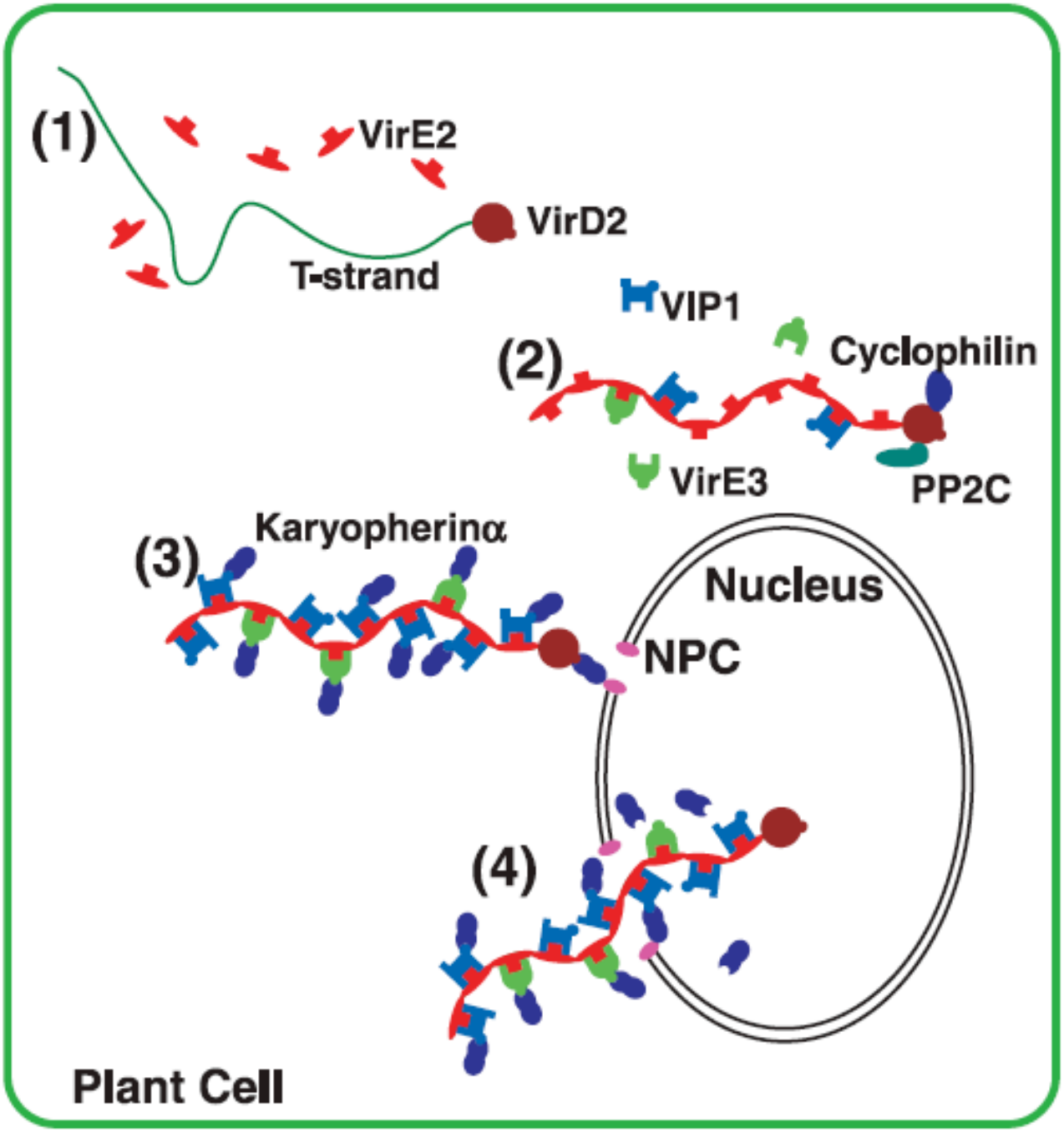


Fig. 1. Summary of major molecular events and structures within the *Agrobacterium* cell that generate the Vir protein machinery and T-strands which then are transported into the plant cell, enter its nucleus and integrate into the genome. The transformation process begins with recognition of plant signals by the bacterial VirA/VirG sensory system, followed by activation of the *vir* loci and attachment of the bacterium to the host cell. The T-strand is excised from the T-DNA region by VirD2/VirD1 and exported, *in cis* with a covalently attached VirD2 molecule and *in trans* with several other Vir proteins, into the plant cell cytoplasm via a VirB/D4 type IV secretion system. Inside the host cell, the VirD2–T-strand conjugate is packaged by numerous molecules of VirE2 to form a mature T-complex. For in-depth discussion on the T-complex transport and nuclear import, and T-DNA integration, see text.


(1)
VirE2
VirD2
T-strand
VIP1
(2)
Cyclophilin
VirE3
PP2C
(3)
Karyopherinα
Nucleus
NPC
(4)
Plant Cell

Beyond the bacterial cell

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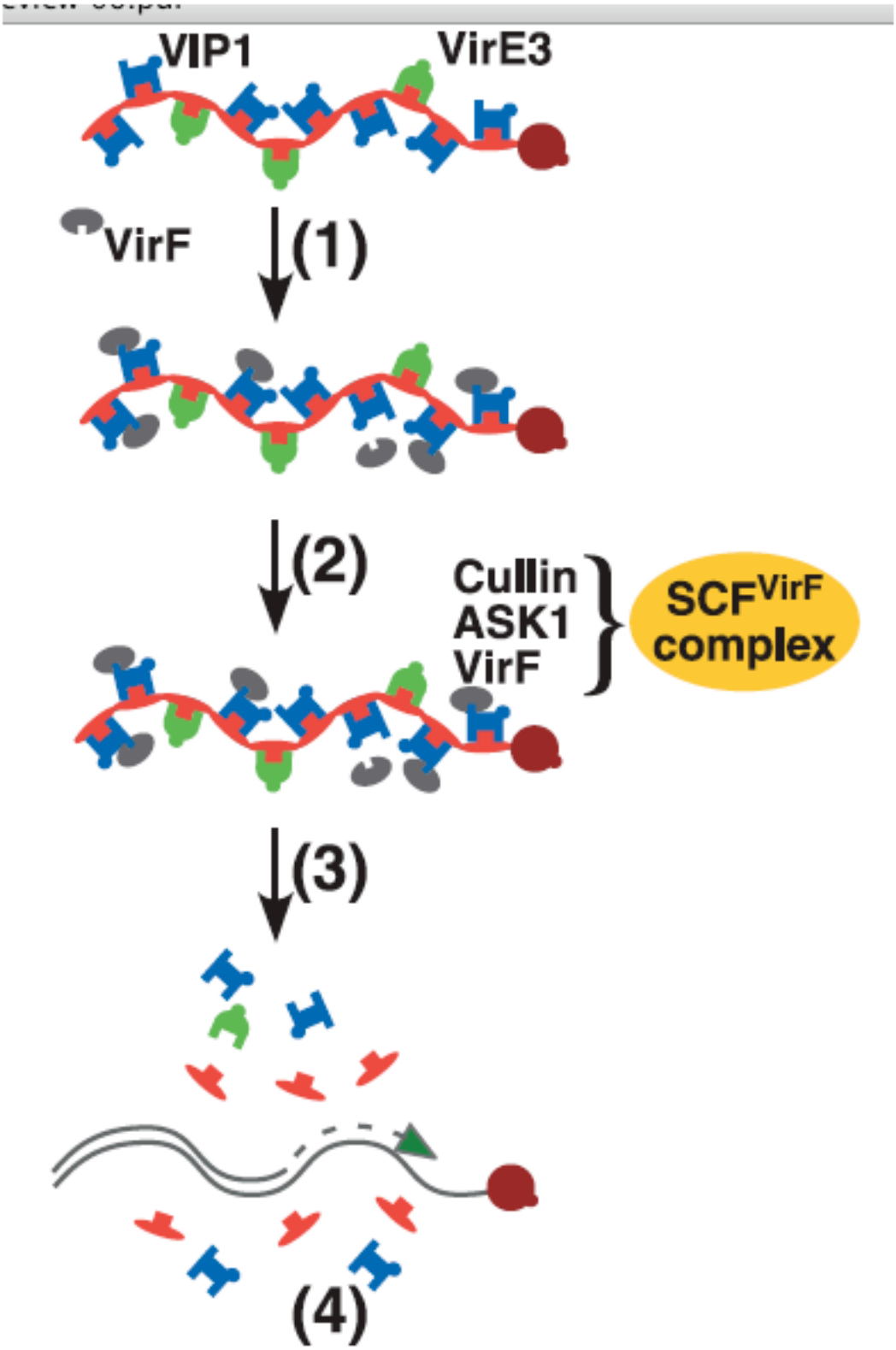
VIP1
VirE3
VirF
(1)
(2)
Cullin
ASK1
VirF
SCF^VirF complex
(3)
(4)

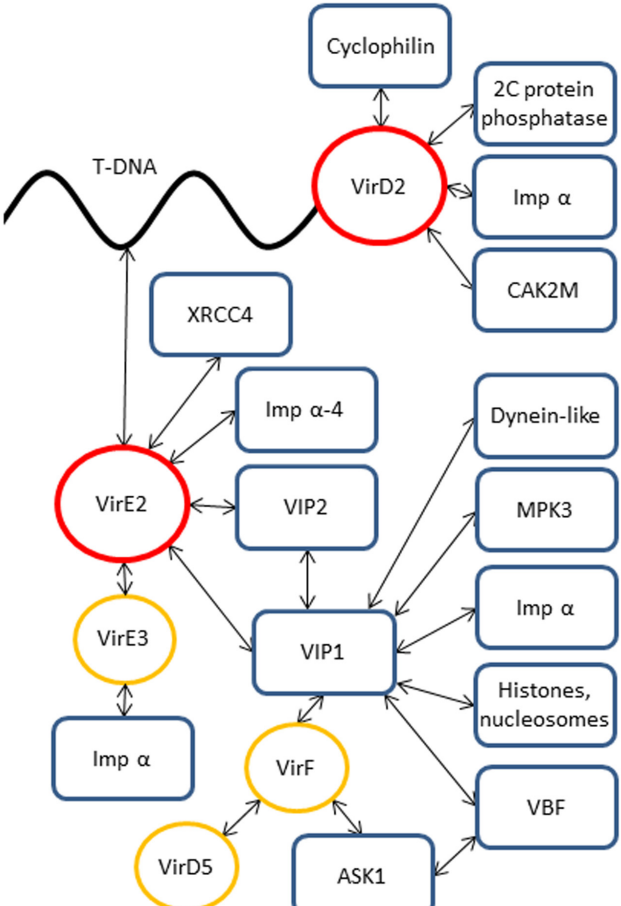

Fig. 3. Network of interactions between translocated *Agrobacterium* effectors and host cell proteins. *Blue rectangles, host factors; yellow circles, bacterial effector proteins; red circles, bacterial effector proteins directly associated with the T-strand. For other details, see text.*

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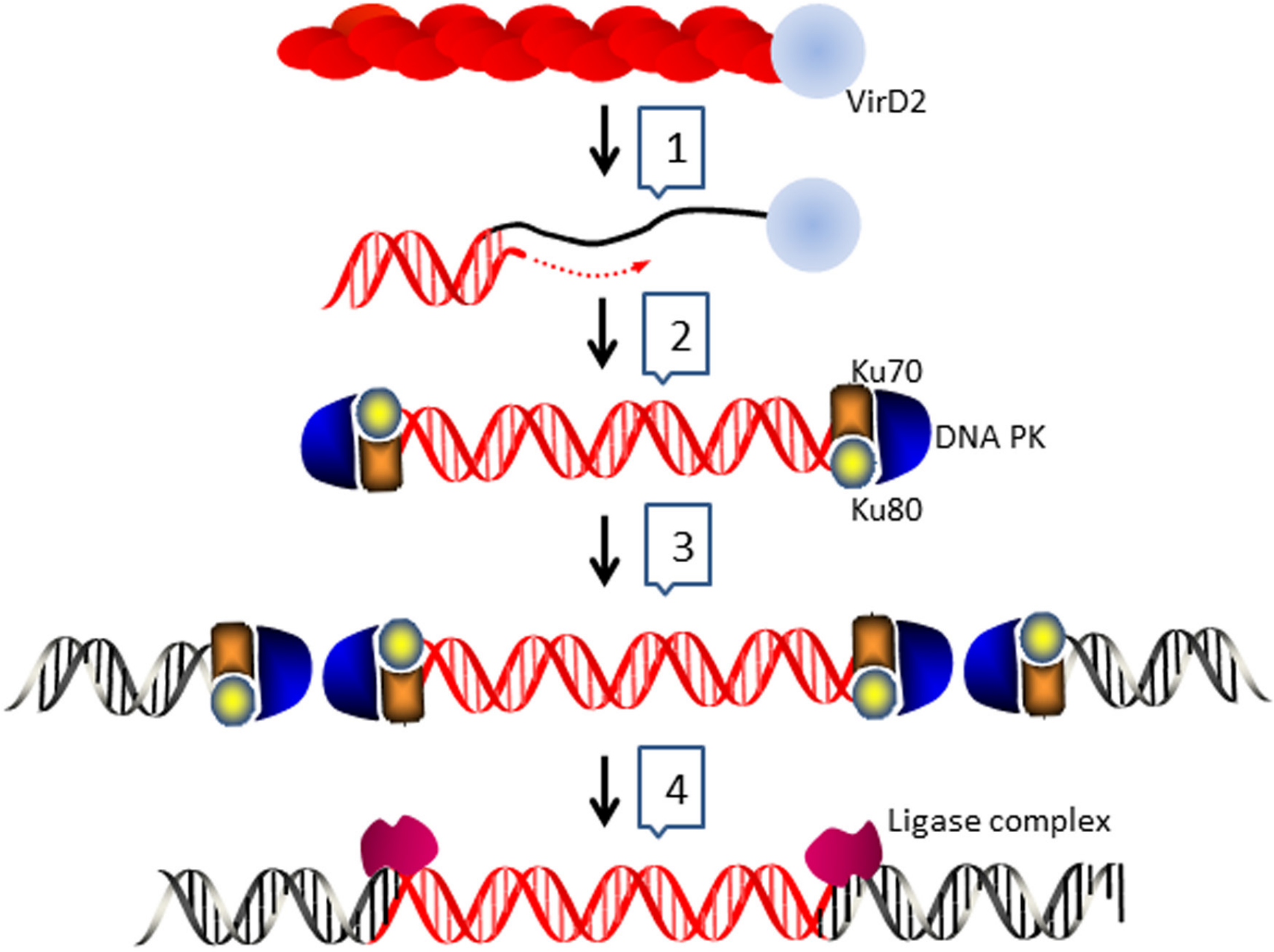

Fig. 4. Model of transferred DNA (T-DNA) integration in host cell chromatin.

Integration of the T-DNA into the nuclear genome

A random process? Or affected by chromatin structure and function?

This is important, since Agrobacterium is an indispensible tool for plant biologists and biotechnologists

Experimental approach – transform plants, identify transformants without applying a selection that requires gene expression (this will reduce or eliminate bias due to the requirement for expression), sequence the insertion sites

Results – no strong bias for any class of genomic position if transformation is done without selection

If transformation is done with selection, there is a bias for transcriptionally active chromatin

A possibly-related observation: overexpression of a linker histone increases transformation efficiencies

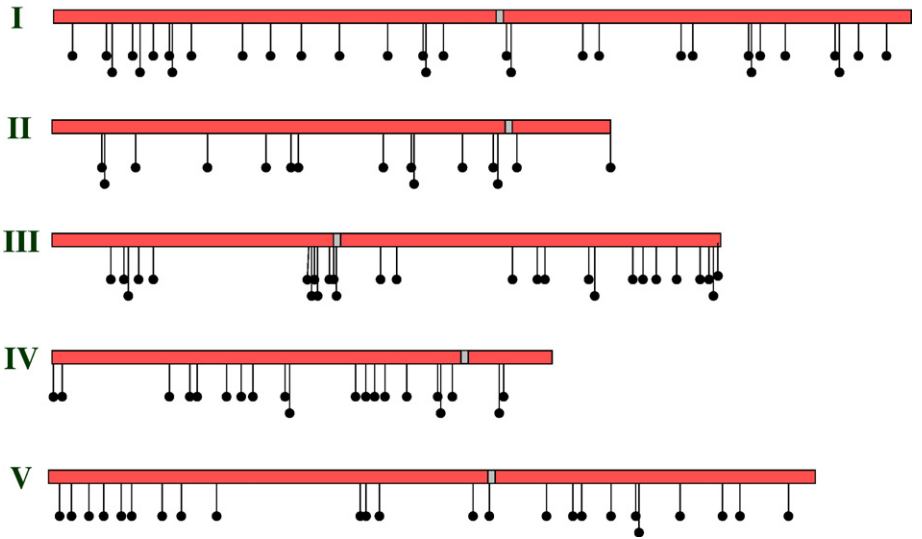

Fig. 1. Chromosomal location of T-DNA insertions in the non-selective library. T-DNA/plant DNA junctions were isolated (using inverse PCR or adapter-ligation PCR) from infected *Arabidopsis* suspension cell cultures that were grown without selection for expression of T-DNA-encoded genes. The T-DNA insertion sites were positioned on the five chromosomes (marked I–V) using TAIR chromosome map tools. Gray bars indicate the sites of centromeres.

Table 1
Classes of T-DNA insertion sites, isolated under non-selective conditions, in the *Arabidopsis* genome

Genic properties	% of junctions isolated under non-selective conditions	Number of junctions isolated under non-selective conditions	Proportion of the total genome (%)
Coding regions	43%	50	44.4%
Exons	32.5%	38	28.8%
Introns	10.3%	12	15.6%
Non-coding regions	57%	67	55.6%
5′ upstream (−500 bp)	9.4%	11	11.0%
3′ downstream (+500 bp)	11.1%	13	11.0%
Intergenic regions	27.4%	32	23.6%
Repetitive sequences [a]	9.4%	11	10.0%
All mapped	100%	117	100%

[a] Includes centromeric and telomeric regions, large rDNA repeats, and 5S rDNA repeats.

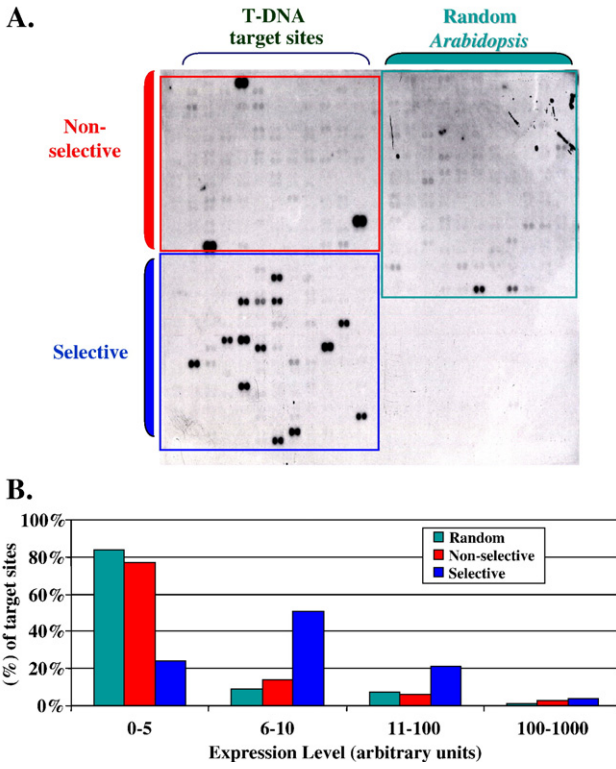

Fig. 2. Expression profiling of T-DNA pre-integration target sites. (A) DNA array containing cloned target site fragments from the non-selective library (upper left quadrant), the selective library (lower left quadrant), and randomly sheared *Arabidopsis* DNA (upper right quadrant) was hybridized with nuclear run-on transcription probes generated from isolated suspension cell nuclei. (B) Graphical representation of the expression levels of the cloned fragments. Note that the over-all expression levels of T-DNA target sites in the non-selective library is similar to that of random *Arabidopsis* DNA fragments, whereas target sites isolated from the selective library are more highly transcribed than are random *Arabidopsis* DNA fragments.

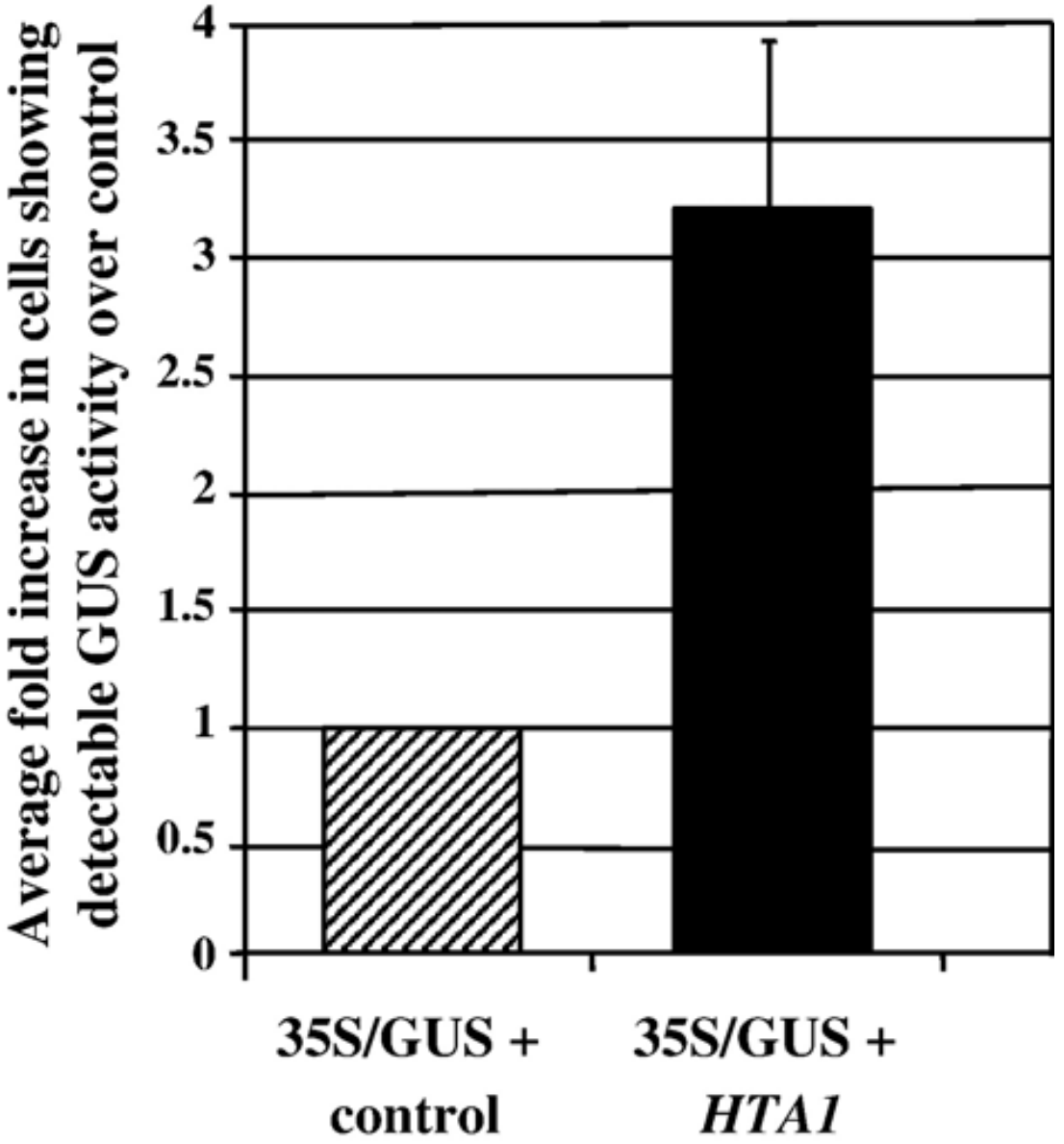

Fig. 3. Expression of a *HTA1* cDNA increases the percentage of transfected cells expressing detectable GUS activity. A *gusA* reporter gene, under control of a CaMV 35S promoter, was co-electroporated into tobacco BY-2 cells with either a control plasmid (an "empty vector" containing the CaMV 35S promoter but no gene), or a plasmid expressing a *HTA1* cDNA under control of a CaMV 35S promoter. Relative GUS activity was estimated by counting the percentage of cells that stained blue with X-gluc. For each electroporation experiment, a minimum of 1000 cells were scored; each experiment was repeated three times. Note that co-expression of the *HTA1* cDNA increases the percentage of cells expressing detectable GUS activity approximately 3-fold.

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Review

Effect of chromatin upon *Agrobacterium* T-DNA integration and transgene expression

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Abstract

Agrobacterium tumefaciens transfers DNA (T-DNA) to plant cells, where it integrates into the plant genome. Little is known about how T-DNA chooses sites within the plant chromosome for integration. Previous studies indicated that T-DNA preferentially integrates into transcriptionally active regions of the genome, especially in 5′-promoter regions. This would make sense, considering that chromatin structure surrounding active promoters may be more "open" and accessible to foreign DNA. However, recent results suggest that this seemingly non-random pattern of integration may be an artifact of selection bias, and that T-DNA may integrate more randomly than previously thought. In this chapter, I discuss the history of these observations and the role chromatin proteins may play in T-DNA integration and transgene expression. Understanding how chromatin conformation may influence T-DNA integration will be important in developing strategies for reproducible and stable transgene expression, and for gene targeting.

Keywords: *Agrobacterium*; Plant genetic engineering; T-DNA; Transgene; Chromatin; Methylation

1. Introduction

Agrobacterium tumefaciens is a Gram-negative soil bacterium which, in nature, incites the tumorous disease Crown Gall on a wide variety of dicotyledonous plants and some Gymnosperms [1–4]. The molecular mechanism of virulence involves the processing of a region of DNA (the transferred, or T-DNA region) from a resident tumor inducing (Ti) plasmid, and the transfer of T-DNA to the plant. In addition to T-DNA transfer, a number of virulence (Vir) proteins are also delivered to the plant cell. These Vir proteins include VirD2 covalently attached to the 5′ terminus of the single-stranded T-DNA (the T-strand), the single-strand DNA binding protein VirE2, and several other proteins such as VirD5, VirE3, and VirF. T-DNA traverses the plant cytoplasm, most likely as a complex with bacterial Vir proteins and plant proteins (the "T-complex"), targets the nucleus, and eventually integrates into the plant genome. Expression of T-DNA-encoded oncogenes results in tumors (for recent reviews, see [5–7]). During the past ~20 years, scientists have harnessed the ability of *Agrobacterium* to deliver DNA to plant cells by first deleting the oncogenes, second by inserting within T-DNA selectable marker genes to identify transformed cells, and third by inserting genes of interest that will confer novel phenotypes upon the transformed plants. Thus, transgenic plants have been generated that express such desirable properties as herbicide tolerance, viral and insect resistance, modified growth and developmental characteristics, and altered nutritional value.

T-DNA transfer and integration into the plant genome requires interaction of the associated Vir proteins with numerous plant proteins. Many of these interactions occur early in the transformation process. For example, VirB2, the major component of the *Agrobacterium* T-pilus, interacts with several membrane-localized plant BTI proteins [8]. VirD2 interacts with the nuclear "shuttle" protein importin α [9], and VirE2 interacts with VIP1, a plant-encoded protein which may also be involved in nuclear targeting of the T-complex [10,11]. Similarly, many interactions of Vir proteins with plant proteins may directly influence T-DNA integration into the plant

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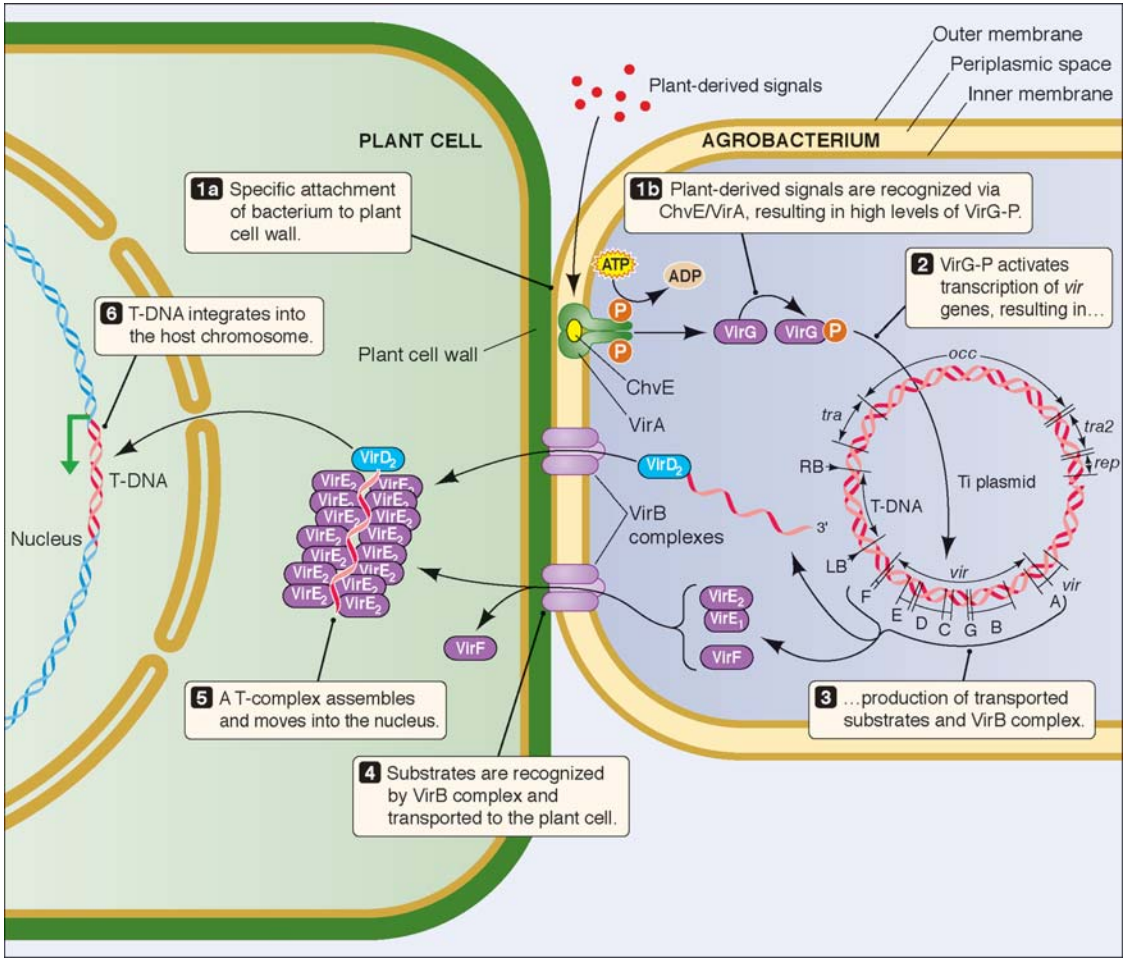

Figure 1

General model of *Agrobacterium*-mediated transformation of a plant cell. See text for details.

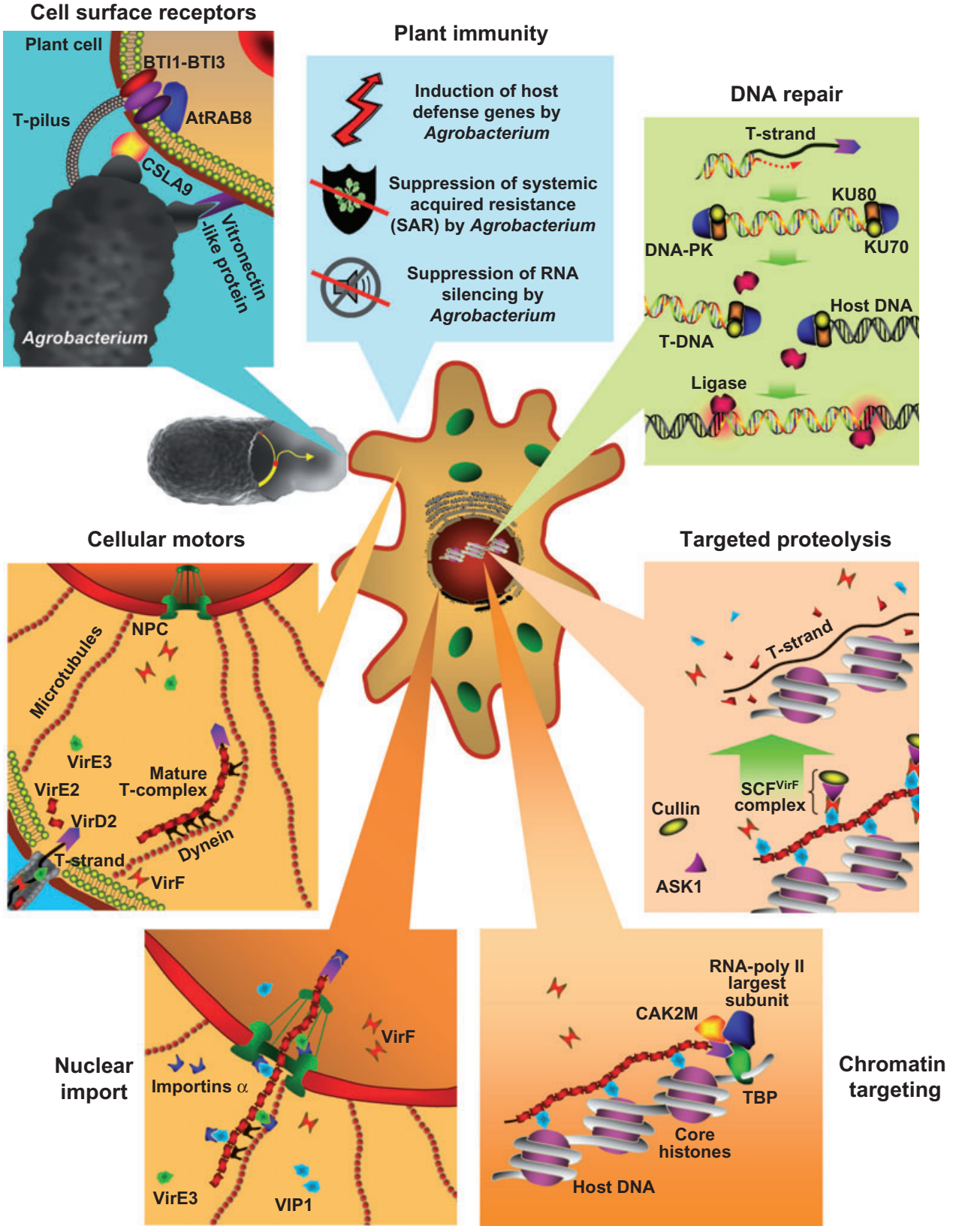

Fig. 2. Summary of major biological systems of the host cell that are involved in the *Agrobacterium*-mediate genetic transformation. Main molecular events associated with each biological system are depicted. For further details, see text.

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Microreview

Biological systems of the host cell involved in *Agrobacterium* infection

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Summary

Genetic transformation of plants by *Agrobacterium*, which in nature causes neoplastic growths, represents the only known case of *trans*-kingdom DNA transfer. Furthermore, under laboratory conditions, *Agrobacterium* can also transform a wide range of other eukaryotic species, from fungi to sea urchins to human cells. How can the *Agrobacterium* virulence machinery function in such a variety of evolutionarily distant and diverse species? The answer to this question lies in the ability of *Agrobacterium* to hijack fundamental cellular processes which are shared by most eukaryotic organisms. Our knowledge of these host cellular functions is critical for understanding the molecular mechanisms that underlie genetic transformation of eukaryotic cells. This review outlines the bacterial virulence machinery and provides a detailed discussion of seven major biological systems of the host cell–cell surface receptor arrays, cellular motors, nuclear import, chromatin targeting, targeted proteolysis, DNA repair, and plant immunity – thought to participate in the *Agrobacterium*-mediated genetic transformation.

Introduction

The ability of *Agrobacterium* to genetically transform a wide variety of plant species has earned it a place of honour in basic plant research and modern plant biotechnology. The transformation results from the production of a single-stranded copy (T-strand) of transferred DNA (T-DNA) molecule by the bacterial virulence machinery, its transfer into the host cell followed by integration into the host genome (for recent reviews, see Gelvin, 2003; McCullen and Binns, 2006). While wild-type *Agrobacterium* species are known as the causative agents of the 'crown gall' disease in a rather limited number of economically important plant species (e.g. Burr *et al*., 1998), recombinant *Agrobacterium* strains are the tool-of-choice for production of genetically modified plants in a very broad range of species (Gelvin, 2003). Furthermore, *Agrobacterium*, at least under laboratory conditions, can transform other eukaryotic species, ranging from fungi to human cells (reviewed in Lacroix *et al*., 2006a), which holds great promise for the future of biotechnology of non-plant species. This remarkably wide host range of *Agrobacterium*, which is in contrast to the relatively narrow host range of many other bacterial pathogens that are typically limited to specific species or genera – raises a question of how the *Agrobacterium* virulence machinery can function in evolutionarily distant and diverse species, crossing the interkingdom boundaries. The answer most likely lies in the ability of *Agrobacterium* to hijack fundamental cellular processes which are shared by organisms of different kingdoms. Thus, the *Agrobacterium*-mediated genetic transformation process relies both on the activity of the bacterial virulence proteins which are required for the early stages of the transformation process (e.g. host recognition and attachment, and T-strand production, Fig. 1), and on the activity of diverse host cellular proteins and systems which are required in the later stages of the transformation process (e.g. nuclear import, integration and expression of the T-DNA, Fig. 1). Here, we summarize the bacterial virulence machinery and then discuss in detail seven major biological systems of the host cell that have been implicated in the *Agrobacterium*-mediated genetic transformation. Our knowledge of these basic cellular functions, which is critical for understanding the molecular mechanisms that underlie genetic transformation of eukaryotic cells, is enhanced using *Agrobacterium* as a unique and powerful experimental tool.

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The roles of bacterial and host plant factors in *Agrobacterium*-mediated genetic transformation

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Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, NY, USA

ABSTRACT The genetic transformation of plants mediated by *Agrobacterium tumefaciens* represents an essential tool for both fundamental and applied research in plant biology. For a successful infection, culminating in the integration of its transferred DNA (T-DNA) into the host genome, *Agrobacterium* relies on multiple interactions with host-plant factors. Extensive studies have unraveled many of such interactions at all major steps of the infection process: activation of the bacterial virulence genes, cell-cell contact and macromolecular translocation from *Agrobacterium* to host cell cytoplasm, intracellular transit of T-DNA and associated proteins (T-complex) to the host cell nucleus, disassembly of the T-complex, T-DNA integration, and expression of the transferred genes. During all these processes, *Agrobacterium* has evolved to control and even utilize several pathways of host-plant defense response. Studies of these *Agrobacterium*-host interactions substantially enhance our understanding of many fundamental cellular biological processes and allow improvements in the use of *Agrobacterium* as a gene transfer tool for biotechnology.

KEY WORDS: *Agrobacterium, genetic transformation, macromolecular transport, T-DNA expression*

Introduction

Agrobacterium tumefaciens has served as an essential tool for research in plant biology and biotechnology in the last several decades (Newell, 2000). The exceptional ability of *Agrobacterium* to transfer a part of its own DNA to the host plant genome represents a rare case of naturally occurring horizontal gene transfer, and is the basis of its use for transgenesis (Gelvin, 2003; Tzfira and Citovsky, 2006). This capability relies on a specialized plasmid, the tumor-inducing (Ti) plasmid, that contains two essential regions required for DNA transfer to the host cell (Fig. 1). The presence of the Ti plasmid is responsible for the virulence of *Agrobacterium*, and a non-virulent strain may become virulent by acquiring this plasmid (Lacroix, 2013a). The first essential region is the transferred DNA (T-DNA) itself; it is delimited by two direct repeat sequences of about 25 base pairs, termed the left and right borders (LB and RB). These borders are necessary and sufficient to define a functional T-DNA element, while the transferred sequence between them may be modified at will. The T-DNA is not transported to the host plant cell as a double-stranded molecule; instead, VirD2 and VirD1, protein products of the Ti plasmid virulence region (see below), form a nuclease that nicks LB and RB, and a mobile single-stranded (ss) T-DNA form, termed the T-strand, is generated by strand replacement synthesis (Gelvin, 2003; Tzfira and Citovsky, 2006) (Fig. 1).

The second essential region, the virulence (*vir*) genes, composed of seven major loci (*virA, virB, virC, virD, virE,virF, and virG*), encodes most of the bacterial protein machinery required for virulence (Fig. 1) (Zupan and Zambryski, 1995). In the wild-type *Agrobacterium*, the T-DNA contains about fifteen genes that are expressed in the transformed plant cells and lead to the crown-gall disease (Escobar and Dandekar, 2003; Lacroix, 2013a). A subset of the T-DNA genes encodes proteins involved in plant growth regulator synthesis and sensitivity, which induce uncontrolled host cell division and result in the visible symptoms of *Agrobacterium* infection, i.e., tumors or crown galls. Other T-DNA gene products are involved in the production of opines, small secreted molecules that *Agrobacterium* cells use as source of carbon and nitrogen (Hooykaas, 1994).

Abbreviations used in this paper: ACC, 1-aminocyclopropane-1-carboxylate; AS, acetosyringone; DIMBOA, 2,4-dihydroxy-7-methoxy-*2H*-1,4-benzixazin-3(*4H*)-one; DSB, double strand break; HR, homologous recombination; IAA, indole acetic acid; MDIBOA, 2-hydroxy-4,7-dimethoxybenzoxazin-3-one; NHEJ, non-homologous end joining; NLS, nuclear localization signal; *rat*, resistant to *Agrobacterium tumefaciens*; SA, salicylic acid; SCF, Skp1-Cullin-F-box protein; SAR, systemic acquired resistance; ss, single stranded; SSGR, single-strand gap repair; T-DNA, transferred DNA; Ti-plasmid, Tumor-inducing plasmid; T4SS, type four secretion system; UPS, ubiquitin/proteasome system; VBF, VIP1 binding F-box protein; VIP, VirE2 interacting protein; *vir* genes, virulence genes.

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