25.7.1 *Agrobacterium*-mediated gene transfer to monocots and dicots

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The interaction of the soil bacterium *Agrobacterium tumefaciens* with plants constitutes a unique kind of genetic flux: the bacterium transfers the T-DNA part of its Ti plasmid to plant cells, where it is integrated into the genome. Possible transfer intermediates, isolated from bacteria and from plants early after transfer, are described. Agroinfection, *Agrobacterium*-mediated delivery of plant viral genomes, is employed to monitor early events in T-DNA transfer in dicot plants. Graminaceous monocots, so far excluded from *Agrobacterium*'s host range because of lack of tumor formation, have been shown to be agroinfectable. This newly discovered interaction between grasses and the pathogen is described in terms of the efficiency of gene transfer as compared with dicot hosts, the involvement of the bacterium's virulence genes, the susceptibility of various developmental stages of the host, the implications for biotechnology, and the evolutionary aspects of this host–parasite relationship.

Key words: T-DNA, agroinfection, maize streak virus, plant transformation, *Zea mays*.

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Les interactions d'une bactérie du sol, l'*Agrobacterium tumefaciens*, avec des plantes constituent une voie unique de flux génique : la bactérie transfère le fragment ADN-T de son plasmide Ti dans les cellules des plantes où il est incorporé dans le génome. Des intermédiaires possibles dans ce transfert ont été isolés des bactéries et des plantes, tôt après le transfert, et sont ici décrits. L'agroinfection, ou le transfert des génomes viraux des plantes par médiation de l'*Agrobacterium*, est utilisée pour suivre les évènements hâtifs liés au transfert de l'ADN-T chez les plantes dicotylédones. Chez les monocotylédones, les plantes de la famille des graminées ont été jusqu'à présent exclues de la gamme d'hôtes de l'*Agrobacterium*, en raison de l'absence de formation de tumeur; toutefois, il a été démontré que ces plantes pouvaient être agroinfectées. Cette interaction de découverte récente entre les graminées et l'agent pathogène est décrite en termes d'efficacité du transfert de gènes par comparaison avec les hôtes dicotylédones, de l'implication de la virulence des gènes de la bactérie, de la sensibilité de l'hôte au cours des stades de son développement, des implications en biotechnologie et des aspects évolutifs des relations hôtes–parasites.

Mots clés : ADN-T, agroinfection, virus de bigarrure de maïs, transformation des plantes, *Zea mays*.

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Introduction

Bacterial symbionts and parasites probably have evolved together with their plant hosts for millions of years. The diversification of modes by which parasites attack their plant victims is enormous; yet among the known nonviral pathogens, only microorganisms of the genus *Agrobacterium* have developed the sophisticated mechanism of genetically and stably transforming their hosts. As a consequence the plant, under the government of the transferred DNA, deviates part of its metabolic resources to build products which only the inciting bacterium can catabolize.

The bacterium only transfers a specific segment of its large Ti (Tumor inducing) plasmid, the T-DNA (Transfer DNA), into plant cells. Analysis of the transfer process has revealed sequences and functions that are required in *cis* and in *trans* to the T-DNA, respectively. The T-DNA is delimited by two almost perfect direct repeats of 25 bp called border sequences. The T-DNA transfer is mediated by products of the virulence region, located on the Ti plasmid. The bacterium does not carry the T-DNA in a constitutively transferable form but has to be induced by wounded plant cells to render its T-DNA movable. A variety of rearranged T-DNA molecules can be detected in induced bacteria. Their structure is described and their possible relevance for the transfer process discussed.

Analysis of possible T-DNA transfer intermediates after passage to plants is technically difficult because of the relatively small amounts of such intermediates, which are difficult to separate from relatively large amounts of T-DNA present in bacterial cells. Thus only forms of T-DNA have been analysed that have already integrated in plant nuclear DNA, and of those only a few down to the level of the base pair. By inserting plant viral sequences between the borders of the T-DNA a system has been established that allows differential detection of plant derived T-DNA molecules. This approach is described and preliminary results are discussed.

The observation of viral symptoms in plants offers a sensitive alternative to the observation of tumors. When a plant is inoculated with an agrobacterial strain carrying a complete or partially duplicated viral genome in its T-DNA, virus symptoms will be produced if the plant is a host for both the bacterium and the virus, a technique called agroinfection. This can in turn be used as a test for agrobacterial host range. Maize, consistently reported to be a nonhost, because of the inability of *A. tumefaciens* to elicit tumors (mostly dicot plants have been reported as hosts so far), has by agroinfection experiments proven to allow at least the delivery of agrobacterial T-DNA. Specificities and implications of this system are described.

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As an introduction to the agrobacterial transfer system, reviews by Gheysen *et al.* (1985), Stachel and Zambryski (1986) and Koukolíková-Nicola *et al.* (1987) should be considered; agroinfection is reviewed by Grimsley and Bisaro (1987).

The mechanism of T-DNA transfer

Analysis of transfer intermediates in the bacterium

Upon induction of agrobacterial strains by cocultivation with plant protoplasts or by pure inducers several T-DNA-derived molecules can be detected: circular molecules (Koukolíková-Nicola *et al.* 1985; Machida *et al.* 1986), single-stranded molecules of lower strand polarity (the so-called T-strands; Stachel *et al.* 1986; Albright *et al.* 1987; F. Dürrenberger, B. Hohn, and Z. Koukolíková-Nicola, to be published), and double-stranded linear molecules (Veluthambi *et al.* 1987; Jayaswal *et al.* 1987; F. Dürrenberger, B. Hohn, and Z. Koukolíková-Nicola, to be published). Most if not all of the circular molecules originally discovered by Koukolíková-Nicola *et al.* (1985) upon closer inspection turned out to be linear molecules with staggered ends: the lower strand cuts are located after the third or fourth base of both 25-bp border sequences and the upper strand cuts are distributed over a range of not more than 25 nucleotides away from the lower strand ones forming predominantly 3′ protruding ends (Z. Koukolíková-Nicola, C. Meduski, and B. Hohn, to be published). The location of the lower strand cuts of the double-stranded molecules is an agreement with results reported earlier (Albright *et al.* 1987; Wang *et al.* 1987). However, no direct comparison of these ends with those of the T-strands is possible since the latter were not precisely mapped.

The enzymatic activity required for the generation of all described molecules is the product of the virulence D locus. Indirect evidence for the double-stranded linear form (F. Dürrenberger, B. Hohn, and Z. Koukolíková-Nicola, to be published) and direct evidence for the T-strand (Young and Nester 1988) indicate that the enzyme remains attached to the 5′ end of the lower strand. It might act as a pilot protein to direct transfer of T-DNA to the plant cell or directly to the nucleus, it may be required for priming of second-strand synthesis, or it may play an important role in integration. The virulence E region was shown to be responsible for another DNA-binding protein, which attaches to single-stranded DNA in a sequence unspecific way (Gietl *et al.* 1987; Das 1988; Christie *et al.* 1988; Citovsky *et al.* 1988). Its function could be production and (or) protection of transfer intermediates.

Figure 1 illustrates schematically the different T-DNA related structures. The relationship between the single-stranded and the double-stranded T-DNA molecules remains to be established. Theoretically, either could be a precursor of the other, but it is not yet clear which of these molecules is actually transferred to the plant. An attractive hypothesis has been put forward for the T-strand transfer: the single-stranded DNA could be mobilized to plants in a manner resembling interbacterial conjugation (Stachel *et al.* 1986; Albright *et al.* 1987). Alternatively, single-stranded or double-stranded molecules could be packaged into some kind of phage coat and transmitted in a viruslike form to the plant. In such a model the requirement for agrobacterial attachment (Matthysse 1987) to plant cells as a necessary step for transformation is not easily incorporated, unless the bacterium–plant contact region constitutes a signal or an entry site for this viruslike particle.

Covalently closed circular T-DNA molecules (Machida *et al.* 1986) are probably not transferred to the plant because of their extremely low abundance and since molecules resembling their structure could not be detected in plants (Bakkeren *et al.* 1989; and see below), and since vectors containing joined border sequences are only poorly transferred to plants (Z. Koukolíková-Nicola, C. Meduski, and B. Hohn, to be published).

Analysis of transfer intermediates in the plant

Figure 2 illustrates the approach: a complete genome of the plant virus cauliflower mosaic virus (CaMV; see Pfeiffer *et al.*, 1987, for a recent review), linearized in its dispensable gene II, is inserted between artificial T-DNA border sequences. Release of this viral T-DNA in the bacterium and transfer to the plant are expected to result in a molecule that, to be able to replicate, express, and systemically spread, requires only circularization (and second strand synthesis, in case the infectious agent is single stranded). Prior integration into the plant genome is not, theoretically, required. Analysis of the resulting border junctions, which are easily cloned out of the large virus population, allows deductions to be made about the molecules entering the plant.

The analysis of a large number of independently arising viruses that have cloned border remnants in gene II showed (Bakkeren *et al.* 1989) that the right end of the T-DNA was found to extend up to the third nucleotide in about one-third of the molecules, whereas much greater variation over the extent of the left border was observed. These observations match with the few analysed T-DNA – plant DNA junctions (reviewed in Gheysen *et al.* 1985, and Koukolíková-Nicola *et al.*, 1987) and further support the validity of the approach, which has the added advantage of recovery of both ends of one T-DNA molecule. In addition, small direct repeats are found in the end joining region of many rescued viral DNAs. In no case was a perfect joined border recovered as would have been expected if a covalently or otherwise tightly closed molecule would be delivered to plant cells.

The host range of *Agrobacterium tumefaciens*

An extensive screen of many plants for tumor formation defined the host range of *Agrobacterium tumefaciens*: of the dicots, about 50% of all tested species proved to be susceptible, whereas of the monocots, only some members of the orders *Liliales* and *Arales* showed some transformation (De Cleene and De Ley 1976; De Cleene 1985). Cereals did not respond.

However, host–parasite interactions are extremely complex, involving many different steps and levels of interplay between host and parasite. The classification of a plant–bacterium relationship depends upon the level at which this association is studied, and hence upon the sensitivity of the assays that are available to investigate the various steps. The invasion of a dicotyledonous plant by *Agrobacterium tumefaciens* involves (*i*) chemotaxis (the bacteria swim towards a wound on the plant up a concentration gradient of substance(s) released by injured tissue (Ashby *et al.* 1987)), (*ii*) bacterial attachment to the plant cell walls, (*iii*) induction of the virulence genes of *Agrobacterium*, (*iv*) processing of the T-DNA, (*v*) transfer of the T-DNA from bacterium to plant, (*vi*) integration of the T-DNA in the nuclear genomes of the recipient plant cells, and (*vii*) expression of genes on the T-DNA in the plant, leading to (*viii*) changes in the phenotype of the plant. The consequence of a successful invasion is a tumor. A sensitive assay for an earlier step, DNA delivery, became available recently with the development of agroinfection (see earlier).

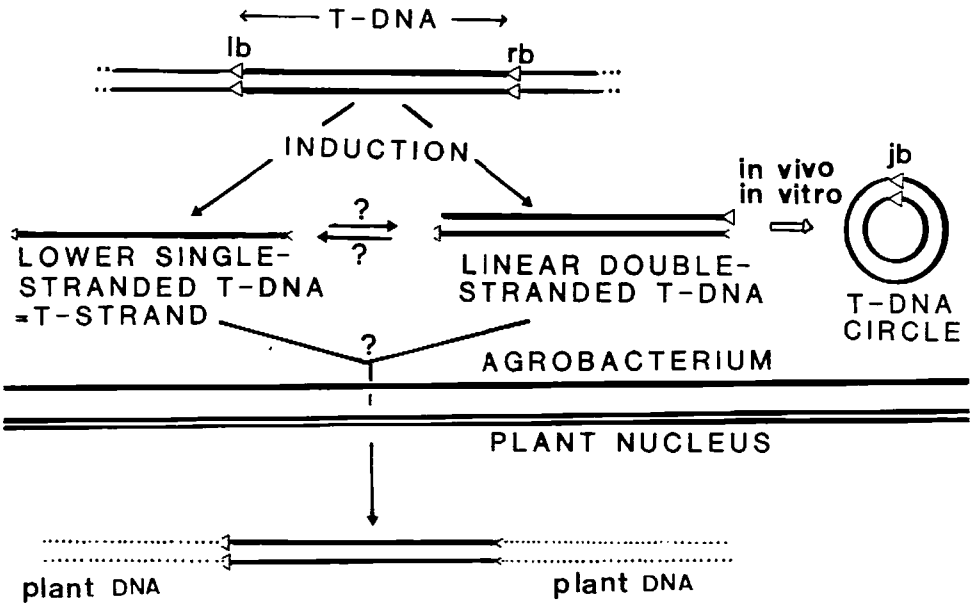

FIG. 1. Schematic presentation of hypothetical relationship between different forms of T-DNA molecules. ◁, border sequence, one per strand; < and ⤙, partial border sequences. *lb*, *rb*, and *jb*, left-, right-, and joined-border sequences.

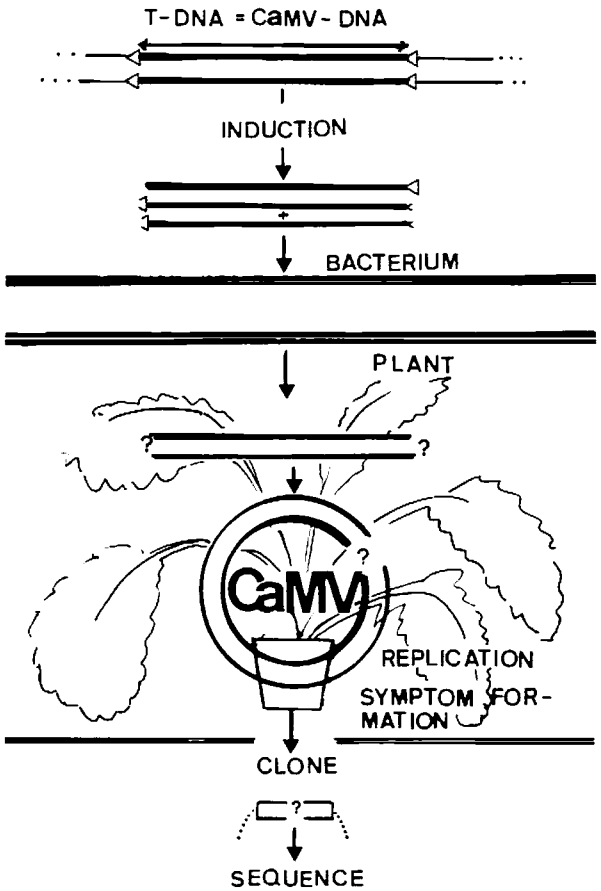

FIG. 2. Illustration of strategy for *in vivo* cloning border "remnants" in CaMV. ? in the lower part of the figure replaces the border sequences (◁, ⤙, <) of the upper part.

Using this technique, gene transfer to the cereal *Zea mays* could be demonstrated. The germinivirus maize streak virus (MSV) was chosen to test this approach, as outlined in Fig. 3. Neither isolated viral particles (route 2, Fig. 3) nor isolated native or cloned DNA (route 3) were ever shown to yield symptoms. The only possible way of viral transmission was *via* the insect vector *Cicadulina mbila*, a leafhopper indigenous to Asian and African countries (route 1). Strains of *Agrobacterium tumefaciens* were constructed carrying dimers of MSV DNA in their T-DNA, and when these were inoculated in the stems of young maize plants (route 5), symptoms developed. Inoculation of the leaves with the same bacterial strains did not incite a viral infection. Control experiments using avirulent strains of *Agrobacterium* did not yield viral symptoms, indicating that the T-DNA transfer mechanism operating in the maize system is probably similar to that described for dicotyledonous plants (Grimsley *et al.* 1987; Hohn *et al.* 1987).

With the establishment of the maize agroinfection system many problems can be addressed, some of which will be discussed here. (*i*) The efficiency of the *Agrobacterium*–monocot interaction is compared with the one of *Agrobacterium*–dicots. (*ii*) The sensitivity of the plant for invasion of the bacterial DNA is assessed as a function of the plant's developmental stage. (*iii*) The requirement of agrobacterial functions for the delivery process is analysed. (*iv*) implications for biotechnology are described. (*v*) Finally, some evolutionary aspects are speculated about.

Efficiency of T-DNA transfer to maize as compared with dicots

To assess T-DNA delivery in a semiquantitative way and to compare it, at least superficially, with dicot transformation, agrobacteria carrying infectious MSV and CaMV (a virus infecting crucifers) units in their T-DNAs were analysed for efficiency of agroinfection as a function of bacterial concentration in the respective inocula. Figure 4 in addition compares these data with tumor formation.

Apart from the actual inoculum size, a multitude of parameters might, singly or in combination, affect efficiencies of symptom formation, for example. (*i*) the immediate environment of the wounded inoculation site could positively (e.g., nutritious compounds) or negatively (inhibitory substances) influence viability of agrobacteria; (*ii*) the availability and (or) efficiency of inducer needed to activate the agrobacterial viru-

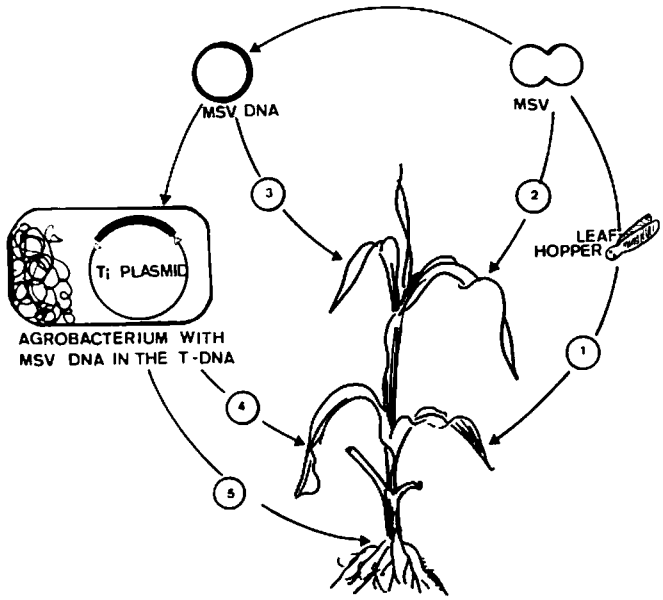

FIG. 3. Life cycles of maize streak virus. See explanations in the text.

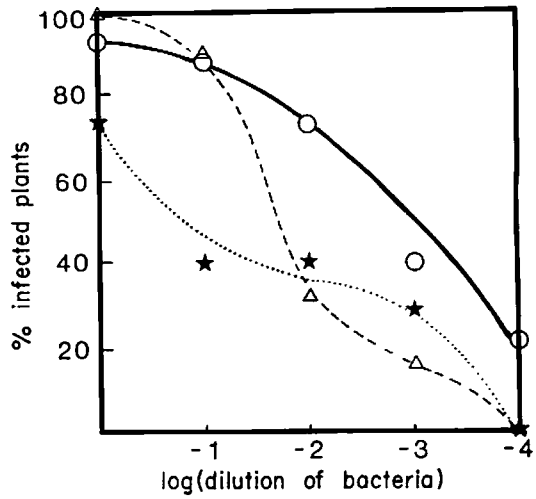

FIG. 4. Comparison of MSV and CaMV symptom and tumor formation on maize and *Brassica rapa* following agroinfection. The inocula consisted of about 10^8 bacteria and dilutions of *Agrobacterium* strain C58 containing an MSV dimer or partial CaMV dimer in a binary vector, respectively. ○, maize symptoms; △, *Brassica* symptoms; ★, *Brassica* tumors. Experimental details are in Grimsley *et al.* (1989).

lence region might vary greatly from plant to plant and within a plant (see below): (*iii*) differences could be the result of varying concentrations and (or) binding constants of "receptors" on the plant, required for agrobacterial attachment. (*iv*) differences in the actual DNA delivery process could be imagined owing to varying permeability of plant cellular compartments, (*v*) plant nucleases could vary enormously between and also within plants as a result of developmental influences, and (*vi*) the transfer intermediate has to be turned into a biologically active form. This requires replication–recombination for MSV, transcription – reverse transcription for CaMV (Grimsley *et al.* 1986), and integration for natural tumor-inducing DNA. The plant's responses to the pathogen's gene expression are then systemic spread and symptom formation for viruses, cell proliferation for tumors.

As a consequence of these considerations a quantitative comparison of the efficiencies of DNA delivery to maize and *Brassica* is difficult. However, we observe that about $10^5 - 10^4$ agrobacteria as actual inoculum still result in viral symptoms in both plants.

On the other hand, symptom and tumor formation in *Brassica* are more directly comparable. This was assayed by inoculating *Brassica* plants with agrobacteria that contained a wild-type Ti plasmid in addition to a binary vector with CaMV DNA in the T-DNA. The efficiencies with which the tumorous and virus symptom phenotypes were observed did not differ significantly (Fig. 4). This was surprising because it has been claimed (but not shown) that agroinfection is much more sensitive than tumor formation (Hille *et al.* 1986); it should be stated however, that another plant species and another *Agrobacterium* strain was used. In other crucifer hosts tested the efficiencies were lower but both tests were again comparable (N. Grimsley, unpublished). We cannot, of course, rule out that agroinfection depends on integration of the viral DNA containing T-DNA.

Sensitivity of different maize plant tissues to agroinfection

The experiments relating to maize described earlier were performed by inoculation of 3-day-old seedlings at the coleoptilar node. Injection of agrobacteria into other parts of such seedlings did not result in symptoms (Grimsley *et al.* 1988). Older plants were found to be susceptible only at the crown and in no case on the leaves. The target for a successful inoculation therefore must be localized in the meristematic region, which has also been documented by microscopical analysis of inoculated plants (Grimsley *et al.* 1988). We do not know which of the many steps (see earlier list) required from the initial bacterium–plant contact to the observed phenotype is dependent on what specific kind of help supplied by the meristematic node tissue.

Agrobacterial functions needed for gene transfer to maize

A number of chromosomal and Ti plasmid encoded functions are needed for virulence (reviewed e.g., in Gheysen *et al.* 1985; Koukolíková-Nicola *et al.* 1987). The former loci have been shown to be needed for agrobacterial–plant attachment, the latter ones for the management of the actual gene transfer from organism to organism.

A set of virulence mutants has been tested for transfer of infectious MSV DNA to maize and compared with a control dicot system (CaMV symptoms in *Brassica*): genes absolutely required for gene transfer to dicots were, not surprisingly, also essential for delivering agrobacterial T-DNA to maize. Interesting variations became apparent in analyzing mutants in the so-called "efficiency" or "host range" loci, virulence E and C (Grimsley *et al.* 1989).

The requirement for the virulence A locus, which most probably functions as a receptor for a plant derived virulence inducer, implies the necessity for a maize-specific inducing compound. Indeed, maize seedling extracts have been shown to contain several inducing compounds (N. Grimsley and J. Oetiker, unpublished). It remains to be established whether any of them bears any similarity to the recently described but not identified inducer isolated from wheat and oat (Usami *et al.* 1988).

Implications for biotechnology

To become an attractive route for genetically engineering

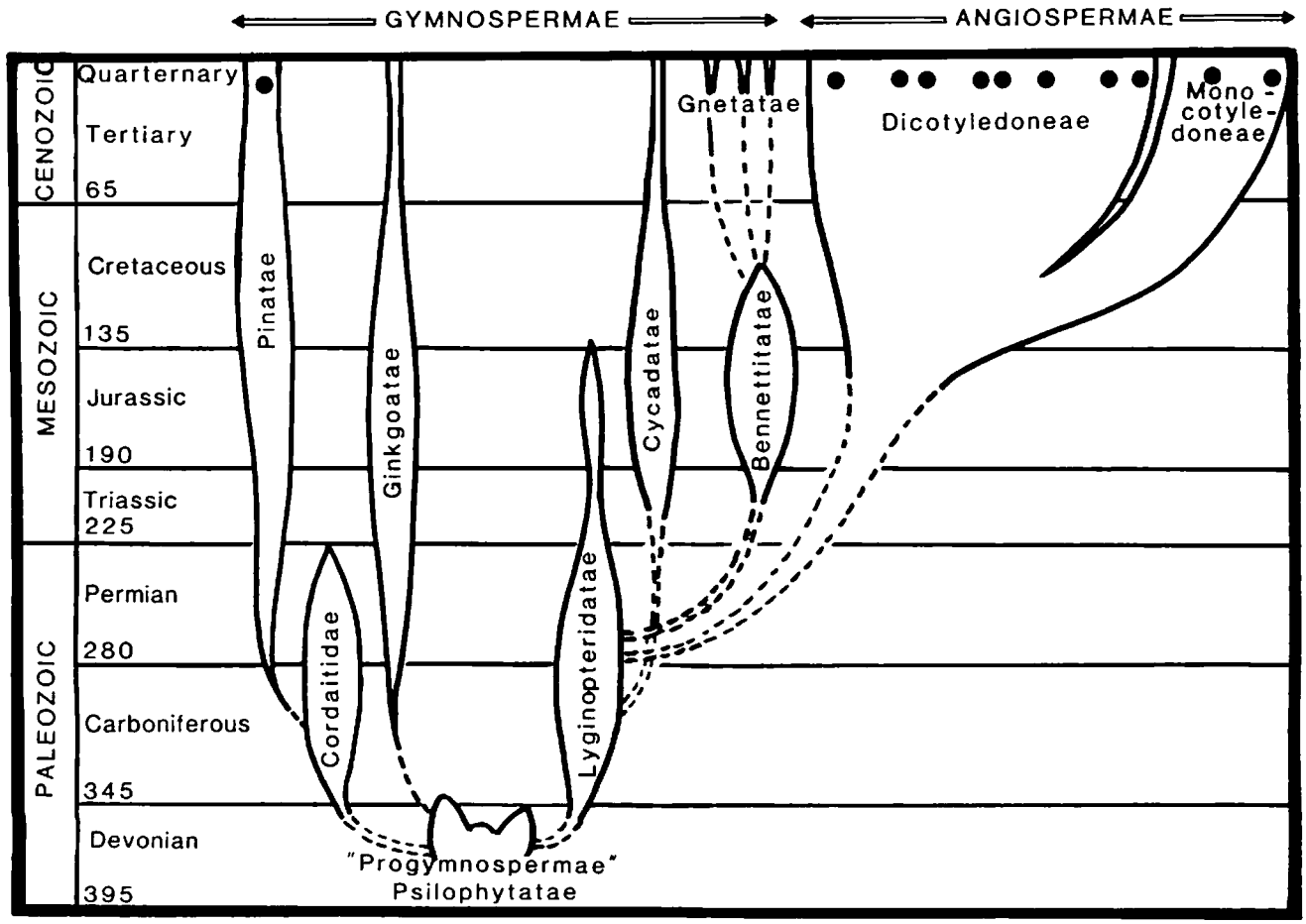

FIG. 5. Phylogenetic tree of spermatophytes, modified after Strasburger *et al.* (1971). ●, documented susceptibility to *Agrobacterium*.

maize and other cereals (see below), stable integration of the foreign DNA into the genome of the host plant and transmission to the progeny have to be demonstrated. Genetic markers other than viral ones are being employed. No principal difficulties are expected, since T-DNA has been shown to integrate into two nongraminaceous monocots (Schaefer *et al.* 1987; Bytebier *et al.* 1987), since non T-DNA related plasmid DNA is readily taken up, integrated, and expressed in maize tissue culture cells (Fromm *et al.* 1986), and since also in dicots T-DNA is integrated at random positions in the genome.

Other cereals that have been shown to be susceptible to *Agrobacterium*-mediated viral inoculation are *Digitaria sanguinalis* and *Avena sativa*, which were tested with digitaria streak virus (Donson *et al.* 1988) and *Triticum*, for which wheat dwarf virus was shown to be agroinfectious (Woolston *et al.* 1988). The test of additional species will depend on the availability of infectious cloned virus isolates for these plants but, by analogy, are also expected to be positive.

Other routes for manipulation of cereals are opening as well: protoplast regeneration of rice (Yamada *et al.* 1986; Abdullah *et al.* 1986; Terada *et al.* 1987) and recently maize (Rhodes *et al.* 1988*a*) has finally been achieved and some transformed but sterile maize plants were described (Rhodes *et al.* 1988*b*). Injection into floral tillers of rye was reported to yield some transgenic plants (De La Peña *et al.* 1987). DNA-coated high-velocity microprojectiles have been used for bombardment of maize suspension culture cells and immature embryos and transient expression of the foreign gene was achieved (Klein *et al.* 1988*a*, 1988*b*). Stable transformation is to be expected. These latter two procedures as well as an *Agrobacterium*-mediated route are attractive because they circumvent difficulties associated with regenerating whole plants from protoplasts.

Evolutionary aspects

How old is the *Agrobacterium*–plant interaction? The first documentations for *Agrobacterium*-mediated DNA delivery to maize came somewhat as a surprise (Graves and Goldman 1986; Grimsley *et al.* 1987). However, phenotypic assays for tumors have been reported for members of the monocot orders *Liliales* and *Arales* (De Cleene and De Ley 1976; Hooykaas-Van Slogteren *et al.* 1984; De Cleene 1985). Molecular evidence including DNA analysis has been presented for *Asparagus*, order *Liliales* (Bytebier *et al.* 1987) and *Dioscorea bulbifera*, order *Smilacales* (Schaefer *et al.* 1987). Thus, DNA transfer to monocots seems more general than originally thought and verification of transfer to cereals only required molecular biologists' tricks of agroinfection. It remains to be established why cereals and possibly other monocots do not respond by producing a tumor, but in the absence of T-DNA

integration data, it is premature to speculate about T-DNA tumor gene expression and hormone balances in cereals.

From the foregoing it seems possible that the evolution of the *Agrobacterium*–plant interaction predates the dicot–monocot divergence. Figure 5 shows one of the classical versions of spermatophyte evolution, with angiosperm appearance and monocot–dicot divergence marked only relatively recently. However, new evidence based on sequence analysis of genes for a glycolytic enzyme supports the hypothesis that angiosperms existed and underwent diversification some 300 million years ago, long before their entry into the fossil record (Martin *et al.* 1989). Plant–*Agrobacterium* interactions may be older still, because a large number of tested gymnosperms were found susceptible (tumor test, De Cleene and De Ley 1976) and recently opine production (Sederoff *et al.* 1986) and even DNA integration (Dandekar *et al.* 1987) were documented. Alternatively, *Agrobacterium* could in a "short" time have became so efficient that it could invade many different plants that had evolved a long time before. However, expression of several T-DNA genes in the plant is necessary for tumor formation and supply of basic resources for the bacterium and it therefore seems unlikely that evolution of this complex system could have occurred rapidly. It seems more likely, that it co-evolved with ancestral plants. Also the discovery, in several species of the genus *Nicotiana*, of T-DNA sequences related to present-day *Agrobacterium* rhizogenes T-DNA points to the actual existence of these phytopathogens early in evolution (Furner *et al.* 1986).

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