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Construction of a gene bank and use of the chromosome walking technique for the detection of new putative agrocin genes in Agrobacterium tumefaciens strain D286

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succinamopine, a structural analog of nopaline (Chilton *et al*, 1984). The plasmids that code for the catabolism of this opine have been called SAP-type Ti-plasmids. In addition to the nitrogenous opines above described, there are phosphorilated sugar derivatives that fit the definition of opine. Such opines were discovered by Murphy and Roberts (1979) while searching for the legitimate substrate of the permease involved in the high affinity agrocin 84 uptake system, and called agrocinopines. Later, a gene determining the permease required for the uptake of agrocinopine A and/or agrocin 84 was identified on the pTiC58 plasmid (Murphy *et al.*, 1981) indicating that agrocinopines are the legitimate substrate of the permease for agrocin 84 uptake, which would explain the high specificity of agrocin 84. Since agrocinopines were detected in crown gall tissue induced by a mutant nopaline strain unable to induce nopaline synthesis in tumors, Ellis and Murphy (1981) defined a third independent functional unit of the nopaline plasmid T-DNA: agrocinopine synthesis, in addition to the existing nopaline synthesis and oncogenicity regions. Messens *et al.* (1985) suggested that the efficient conversion of phosphate to agrocinopine A and its secretion from tumor cells is probably advantageous for the associated bacteria since a phosphorus-containing nutrient would become available in addition to the nitrogen-rich nopaline.

In addition to the nutritional advantage conferred by agrocinopine A it has been shown that certain agrocinopines (A and B) but not nopaline promote the transfer of nopaline type Ti-plasmids (Ellis *et al.*, 1982). Octopine and the related compounds octopinic acid and lysopine promote transfer of octopine Ti-plasmids (Petit *et al.*, 1978; Klapwijk *et al.*, 1978).

Since almost all wild type Ti-plasmids specify opine synthesis they may be considered as a key feature in crown gall disease and the ecology of the Ti-plasmid. Opines are the substrate of Ti-encoded catabolic path-

ways, therefore they provide an ecological advantage to the Ti-plasmid in terms of nutrient supply . Opines also promote conjugal transfer of the Ti-plasmid, which therefore takes place in an established ecologial niche. Production of opines has been appropriately suggested to be the biological rationale for the existence of crown gall tumors.

1.3.5 ROLE OF TI-PLASMIDS IN DETERMINING THE HOST RANGE OF CROWN GALL

Virulent strains of *A. tumefeciens* can be classified based on the host plants on which they can incite tumors. For example strains carrying the octopine Ti-plasmid pTiA6 induce galls in a broad range of hosts (De Cleene and De Ley, 1976) in contrast, those carrying the octopine Ti-plasmid pTiAg63 have a narrow-host- range restricted almost exclusively to grapevines (Panagopoulos and Psallidas, 1973; Thomashow *et al.*, 1980).

Some of the early evidence that the Ti-plasmid is responsible, at least in part, for the host range of virulent *A. tumefaciens* strains came from the work of Loper and Kado (1979). They extended the host range of *A.tumefaciens* 1D1109 (known to induce crown gall only on grapevines) to include many plant species by transferring a Ti-plasmid from a broad-host range pathogen. Transconjugants induced tumors on the same 28 plant species as those of the original Ti-plasmid donor strain ID1 (pRK2)(pTi). In addition, Thomashow *et al.*, (1980) transformed strain A136, which is a broad-host-range strain that had been cured of its Ti-plasmid while retaining its cryptic plasmid, with the Ti-plasmid pTiAg63 from the narrow-host-range strain Ag63. Strain A136 was unable to induce tumors on the test plants whereas the transformants showed the same narrow host range than strain Ag63, forming large active galls on grapevines. These authors also reported to have found considerable differences in the endonuclease restriction pattern between narrow and broad-host-range octopine Ti-plasmids.

Depicker *et al.*, (1978) and Knauf *et al*, (1983) showed that even distantly related Ti-plasmids contain DNA sequences that are closely related to the oncogene region of the broad-host-range pTiA6. The general rule of highly conserved T-region DNA sequences does not hold with the pTiAg63 plasmid family, where the DNA homology between these limited-host-range Ti-plasmids and the TL-DNA region of the pTiA6 plasmids is barely detected under standard hybridisation conditions (Thomashow *et al.*, 1981). With this work as background Buchholz and Thomashow (1984) postulated that there were specific regions of the Ti-plasmid accounting for the host range of virulent *A. tumefaciens* strains. As a first step to identifying such host range-specifying regions in Ti-plasmids they compared the oncogene complements of *A. tumefaciens* Ti-plasmids with limited (pTiAg63) and broad (pTiA6) host ranges and also defined the regions of the pTiAg63 that are transferred to plant cells. Their results showed that pTiAg63 has DNA sequences related to most of the genes encoded by the oncogene region, the TL-DNA, of pTiA6 and that these sequences are divided between two T-DNA regions: (i) the TA-DNA, which encoded sequences related to pTiA6 genes 4, 6a, 6b, and a portion of gene 3 and (ii) the TB-DNA, which encodes sequences related to genes 1 and 2. Buchholz and Thomashow observed that although pTiAg63 has DNA sequences related to most of the genes of the TL-DNA of pTiA6 the two plasmids incited tumors with different morphologies, a clear indication that the overall effects of the pTiAg63 and pTiA6 oncogenes differ. In contrast to the tumors incited by pTiA6 (unorganised callus) the plant tissues transformed with pTiAg63 grew as clumps of root-like structures. This is typical of a relatively high auxin to cytokinin ratio and indicative of inactivation or inefficient expression of oncogene 4 (*cyt*). These observations together with findings by Garfinkel *et al*. (1980; 1981), who reported that agrobacteria harboring pTiA6 derivatives with a mutated oncogene 4 incite tumors in fewer hosts than do strains harboring the wild-type Ti-plasmid, lead Buchholz and Thomashow to postulate that if the oncogene 4 sequences of pTiAg63 are inactive, at least in some plant species, it could explain

the characteristic host range of this plasmid. In order to support this hypothesis buchholz and Thomashow (1984 b) constructed derivatives of pTiAg63 containing pTiA6 oncogenes 4, 6a, and 6b in the TB-DNA region and showed that strains harboring these pTiAg63 derivatives incited unorganised tumors on *Nicotiana rustica* whereas agrobacteria harboring pTiAg63 incited rooty tumors. Strains harboring pTiAg63 derivatives were not able to induce galls in some of the test plant species in which pTiA6 incited tumors. Therefore , Buchholz and Tomashow (1984 b) have suggested that factors other than oncogene 4 sequences also contribute to the different host range characteristics of pTiA6 and pTiAg63.

Additional evidence supporting the role of oncogene 4 in determining host range was obtained by Hoekema *et al.*, (1984). They extended the host range of *A. tumefaciens* strain LBA649 (pTiAg57), limited to grapevines and a few other species, through the introduction of the T-region from the wide host range octopine plasmid pTiAch5. The genes responsible for the host range extension were then detected using site directed mutagenesis. Hoekema *et al.* (1984) found that inactivation of the *cyt* gene abolished the capacity of the T-region to extend the host range of LBA649. They then cloned the *cyt* gene into a disarmed T-region plant vector and used it in complementation studies with pTiAg57 via the binary vector strategy (Fraley *et al.*, 1983) and showed that the presence of the *cyt* gene from a wide-host-range Ti-plasmid was sufficient to expand the host-range of the narrow-host-range.

Although the addition of the wide-host-range TL-DNA (Knauf *et al.*, 1983), and specifically the addition of the wide-host-range *cyt* gene (Buchholz and Thomashow, 1984; Hoekema *et al.*, 1984) expands the host-range of a narrow-host-range strain, the resulting host range is less than that conferred by the donor wide-host-range Ti-plasmid. The *cyt* gene hypothesis does not explain, as well, the fact that certain *Vitis* cultivars which are susceptible to limited-host-range *Agrobacterium* strains are

resistant to tumor formation by wide host range strains (Thomashow *et al.*, 1980; Knauf *et al.*, 1982). Other factors, in addition to *cyt* gene expression must play a role in determining host range. In this regard, Yanofsky *et al.* (1985) reported that in addition to the *cyt* gene, two genes from the virulence region of pTiA6 (*vir* A and *vir* C) must be introduced into a limited host range strain harboring the plasmid pTiAg162 in order to restore a wide host range phenotype. Recently a chromosomal virulence region (Douglas *et al*, 1985) and a chromosomal locus regulating Ti-plasmid virulence genes have been identified adding new elements which may play a role in host range.

Biotypes or biovars must be included when considering factors affecting host range of crown gall but the information available is difficult to interpret. Biochemical characteristics on which biotypes are based are not determined by the Ti-plasmid, yet there is correlation among the three biotypes actually recognised and their host ranges. On the other hand the capacity of the *cyt* gene of a broad-host-range Ti-plasmid to expand the host range of limited-host-range-strains implies that the biotype cannot determine the host range of pathogenic agrobacteria. Thomson (1985), stated the need to study the ecology of the three biotypes with or without Ti- plasmids, in soil and on root and crown surfaces. Since sensitivity to agrocin can be affected by both Ti-plasmid and strain biotype, this information is vital to the effective implementation of biological control of crown gall (Thomson, 1985).

Although mostly under laboratory conditions, some aspects of relevance to the host plant-agrobacteria ecological interactions have been studied. Some insight has been gained, for example, into the lipopolissacharide (LPS) mediated adhesion of *Agrobacterium* strains (New *et al.*, 1983). Loss of adhesion leads to suppression of tumorigenicity (Farrand *et al.*, 1981) and the ability to produce site-binding LPS can be transferred with the Ti-plasmid but this character can seemingly be determined by other bac-

terial information as well (Whatley *et al.*, 1978). It is possible that adhesion may play a role in determining host range but it is difficult to postulate a mechanism that would act at a very specific level.

Some progress has been made in the elucidation of the plant molecular signals that induce expression of the *A. tumefaciens* loci needed for virulence (Stachel *et al.*, 1985; Stachel and Zambrisky, 1986; Bolton *et al.*, 1986). The work of the above mentioned authors has shown that certain plant signal-molecules activate T-DNA transfer in *A. tumefaciens*. Bolton *et al.* (1986) identified seven commercially available plant-derived phenolic compounds that were able to induce the virulence loci of *A. tumefaciens* strains. In a more ecological approach Stachel *et al.* (1985) worked with exudates of wounded plants. They showed that two phenolic compounds (acetosyringone and α-Hydroxyacetosyringone) induce the entire *vir* regulon in *Agrobacterium* as well as the formation of T-DNA intermediate molecules. This, they postulated, would allow *Agrobacterium* strains to recognise susceptible cells in nature. Further work carried out in the same laboratory (Stachel and Zambrisky, 1986) showed that the pTiA6 *vir* loci are organised as a single regulon whose induction by plant signal-molecules is controlled by *vir* A and *vir*G. Stachel and Zambrisky (1986) proposed that *vir* A encodes a transport protein for the plant signal-molecule, and *vir* G a positive regulatory protein that together with the plant molecule activates *vir* expression. It is interesting to recall the findings of Yanofsky *et al.* (1985), namely that in addition to the *cyt* gene, genes *vir* A and *vir* G of pTiA6 had to be introduced into a limited-host-range strain carrying pTiAg162 in order to restore a wide-host-range phenotype. Since *vir* A encodes a transport protein for the plant signal-molecule it can be hypothesised that pTiAg162 was unable to respond to a specific plant signal.

The recognition of plant molecular signals and the response to them by *A. tumefaciens* is likely to be a new element playing a role in determi-

nation of host range. Homology studies of the *vir* region of Ti-plasmids with different host ranges difficult to explain by the *cyt* gene hypothesis are an important first step to determine if elements of recognition play a role in host range. For example, the limited host range of strains carrying pTiAg63 could be a reflection of a *vir* region able to recognise and/or respond to a limited spectrum of plant molecular signals. If this is so, DNA homology studies between pTiA6 and pTiAg63 should indicate differences in the DNA sequences of their virulence regions.

1.3.6 THE TI-PLASMID AS A PLANT GENE VECTOR

The potential of the Ti-plasmid of *A. tumefaciens* for the introduction of genes into higher plants was postulated when it was shown that the Ti-region becomes integrated into the nuclear DNA of plant cells (Chilton, 1977). Since then every major step taken towards the elucidation of the structural and functional organization of the Ti-plasmid has been, in some way, instrumental in the improvement of Ti-plasmid-derived plant vectors. The general characeristics that an efficient gene vector for the genetic engineering of higher plants should possess have been defined by the pioneers in the field, Schell and Montagu(1983). Basically the gene vector should provide an easy and efficient system for the introduction of genes into the genome of plant cells and the foreign genes should be expressed in the transformed cells and stably transmitted to the offspring of the initially transformed cells. In addition an ideal gene vector should also make it possible to introduce, maintain and express genes of diverse origins. Finally the gene vector should have a very broad host range. pTi-derived vectors for transferring genes into higher plants are not necessarily useful vectors for the genetic engineering of higher plants. Of the several pTi-derived vectors that have been developed for the transfer of genes into higher plants only two types are useful for genetic engineering purposes.

Intermediate vectors

The first plant vector system based on a Ti-plasmid (Leemans *et al.*, 1981) was designed with the purpose of studying aspects of the molecular genetics of crown gall , particularly to construct a functional map of T-region DNA utilising site specific mutagenesis. The intermediate vector constructed by Leemans *et al.* (1981) contained specific Ti-plasmid sequences and were capable of replication in both *E coli* and *A. tumefaciens*. Isolated DNA fragments could then be introduced *in vitro* into predetermined sites of the T-region-derived fragment. Then, site-specific inserts and/or deletions-substitutions in Ti-plasmids were produced by exchange of the modified T-region sequences for the wild-type sequence by *in vivo* recombination between intermediate vectors and resident Ti-plasmids. Co-integrates could be discriminated from mutated pTi (double cross over) by selectable markers. Leemans *et al.* (1981) showed, using these intermediate vectors, that the foreign DNA fragments in the T-region were co-transferred without rearrangements, a very important requisite for plant genetic engineering. At this stage, however, oncogenes were being transferred along with the inserted foreign DNA, a major drawback since it was not possible to regenerate normal plants from tumorous growths.

Mutant pTi vectors

Following the development of intermediate vectors important facts of crown gall disease were revealed, among them; (i) the functions encoded by the T-DNA that prevent normal differentiation were deciphered and (ii) the T-region DNA sequences which define the border regions of the transferred DNA in nopaline and octopine Ti-plasmids were accurately determined, and their function established. Capitalising on these findings, Zambrisky *et al.* (1983) constructed the first Ti-plasmid vector for the

introduction of DNA into plants cells without altering their normal regeneration capacity.

The deletion vector pGV3850 developed by Zambrisky *et al.* (1983) has the following outstanding features. (i) T-region DNA border sequences, (ii) no genes which prevent normal differentiation of transformed cells and (iii) it has a genetic marker (the nopaline synthase gene, which maps immediately adjacent to the right T-region DNA border) to monitor the presence of T-DNA. pGV3850 contains, between the 2 T-region borders, pBR322. Therefore, besides insertion into it of defined clones containing a pBR322 sequence by a single cross over event this vector can be used as a recipient for cloned banks of DNA in pBR322 or its derivatives in "shot gun cloning". The total population of hybrid plasmids in *Agrobacterium* can be used to infect plant cells and can be subsequently screened for expression of the genes of interest. In addition, the development of the *ColE* mobilisation system for direct mobilisation of plasmids containing pBR322 sequences to *Agrobacterium* (Van Haute *et al.*, 1983) eliminates the subcloning step required by the intermediate vectors developed by Leemans *et al.* (1981).

Chimaeric genes

Herrera-Estrella *et al.* (1983) have constructed plasmids for the expression of genes behind the nopaline synthase (NOS) promoter. They had unsuccessfully attempted to obtain expression of foreign genes introduced, and showed to be integrated, into the tobacco genome using Leemans et al. (1981) intermediate vectors. They attributed their failure to the possibility that the plant transcription mechanism was unable to recognise the transcription signals (promoter sequences) carried by these foreign genes. In order to overcome these expression barriers Herrera-Estrella *et al.* (1983) constructed a number of chimeric genes consisting of (i) promoter sequences derived from the nos gene and (ii)coding se-

quences d rived from foreign genes. They induced tumors in tobacco seedlings and the tumors had the activity of the foreign gene inserted in the chimeric gene. Thus, foreign genes are not only transferred but also functionally expressed when the appropriate constructs are made, using promoters known to be active in plants. The NOS promoter is particularly useful because of its wide host range. It is functional in calli derived from most dicotyledoneous plants so far tested (Herrera-Estrella *et al.*, 1983) and it also appeared to be expressed in most tissue of regenerated NOS-containing plants.

The vectors described so far in this discussion are useful tools for studying the molecular biology of crown gall but their application to plant genetic engineering is limited. In some cases there is tumorous growth associated with the integration of the genes of interest (Herrera-Estrella *et al.*, 1983; Leemans *et al.*, 1981) or integration of pBR322 sequences in the transformed cell (Zambrisky *et al.*, 1983). These two problems were overcome with the development of two powerful generations of vectors, the split end vectors (SEV) and the binary vector system.

Split end vectors (SEV)

In the SEV system constructed by Fraley *et al.* (1985) they used a derivative of an octopine Ti-plasmid. In this acceptor plasmid all the TL-DNA oncogenic functions, the TL-DNA right border and all of the TR-DNA were deleted and replaced with a kanamycin antibiotic resistance marker. The resulting a-virulent plasmid contained only the TL-DNA left border sequence and a region of homologous DNA to allow recombination with intermediate vectors such as pMON200. Following conjugal transfer of pMON200 into *A. tumefaciens* cells recombination via the homologous LIH (left inner homology) regions results in the production of a co-integrate plasmid called SEV because the T-region DNA border sequences are present on sep-

arate plasmids. The SEV system is powerful and versatile for plant transformation experiments because of the following properties. (i) Fully functional a-virulent T-region DNA is formed via a single cross over. (ii) Transfer of such DNA can be monitored by both selectable (kanamycin resistance) and scorable (NOS, carried by the intermediate vector pMON200) marker genes. (iii) Finally, pMON200 (intermediate vector) contains a synthetic DNA multilinker with several unique restriction sites for insertion of foreign DNA sequences. The SEV system has been tested by introducing and expressing a number of genes of different origin in plants (mouse dehydrofolate reductase, human chorionic gonadotropin, bacterial hygromycin phosphotransferase and chloranphenicol acetyl transferase, and soybean 7s storage protein, among others) (Fraley *et al.*, 1985).

Binary or shuttle vectors

Binary vectors are the most perfectioned type of vector currently available for genetic engineering of higher plants. Their name derives from their capacity to replicate in both *A. tumefaciens* and *E. coli* in addition to being easily mobilised between these bacterial strains. The development of these vectors took place as a consequence of the finding thatthe T-region DNA does not have to be physically linked to the virulence region of the Ti-plasmid in order to be transferred (Stachel *et al.*, 1985) and this can be achieved as long as virulence region and T-region are maintained in the same cell. It follows that there is no need for recombination of the plant transformation vector into a resident Ti-plasmid. This has allowed the construction of vectors which can be used for cloning the fragments of interest in *E. coli*,and mobilised to an adequate *Agrobacterium* strain which provides the *vir* functions in *trans* in order to promote transfer of the binary vector to receptive plant cells. The binary vector pEND4K developed by Klee *et al.* (1985) has several outstanding features that make it a powerful and elegant plant vector. pEND4K

was derived from a wide host range derivative which contained an origin of replication an origin of transfer and a neomycin phosphotransferase (NPTI) gene. pEND4K has no internal T-region DNA sequences, it only has the left and right borders derived from the TL-DNA of pTiA6.

In order to detect transformed cells , Klee *et al.*, (1985) incorporated a chimeric gene which confers kanamycin resistance on transformed plant cells (Fraley *et al.*, 1983). Another important feature of pEND4K is that it allows the cloning of large fragments because it contains the lambda cohesive ends . pEND4K also has the alpha complementation sequence of the PUC series plasmids (Vierra and Messing, 1982) which facilitate cloning. pEND4K retains the ability to screen for inserts into the unique restriction sites on X-gal plates, the high copy PUC plasmid replication origin and the gene conferring chloramphenicol resistance (Klee *et al.*, 1985). In addition, the presence of the PUC plasmid replication origin results in plasmid yields five times greater than that of constructs lacking this origin (Klee *et al.*, 1985).

It is clear that the development of pTi-derived vectors for the genetic engineering of plants will have a great impact in plant biotechnology (Schell and Montagu, 1983, have discussed such potential). The host range of Ti-plasmids, unable to transform most of the economically important plants, namely cereals, together with the fact that only few species of plants can be regenerated from single protoplasts (which are easily transformed with naked DNA) are the current bottleneck in plant genetic engineering. Use of sophisticated vectors, therefore awaits developments in these areas to fulfil their potential in biotechnology. Basic research in plant molecular biology will certainly benefit from pTi-derived plant vectors.

1.4 BIOLOGICAL CONTROL OF CROWN GALL

1.4.1 EARLY WORK ON A. RADIOBACTER STRAIN 84.

The first evidence suggesting the feasibility of biological control of crown gall dates back to 1960 when Stonier reported the discovery of an antimicrobial agent, non proteinaceous in nature, active against a variety of pathogenic and non-pathogenic *A. tumefaciens* strains. The antibiotic was named "agrobacteriocin 1" because the history of its discovery and its specificity characteristics resembled those of the bacteriocin colicin.

Although it opened a new field in crown gall research, agrobactriocin 1 was no found suitable for biological control. In 1972, however, the first *Agrobacterium* strains that were able to inhibit crown gall formation were isolated (New and Kerr, 1972; Kerr, 1972). Among the isolates was *A. radiobacter* strain 84 (New and Kerr, 1972), a non-pathogen isolated from soil round a peach gall. When peach seedlings were potted in soil artificially inoculated with strain 84 and a pathogenic *A.tumefaciens* strain, both of biotype 2, 100% inhibition of gall formation was achieved (New and Kerr 1972). Dipping of seeds in a suspension of strain 84 prior to potting in soil inoculated with the pathogen *A. tumefaciens* strain also decreased gall formation (Kerr, 1972) .The efficiency of strain 84 in the biological control of crown gall of stone fruit was so remarkable that in less than 2 years the practice of inoculating stone fruit seeds and roots with strain 84 became widely spread in Australia (Kerr, 1974). The mechanism of biological control, however, was far from clear. Any mechanism proposed would have to account for the specificity of interaction between the pathogenic strain and strain 84. In addition, little was known about the causative agent of crown gall, believed by some authors (New and Kerr, 1972) to be a temperate phage.

Shortly after the isolation of *A. radiobacter* 84, Kerr and Htay (1973) provided evidence that a bacteriocin-like compound that they termed

bacteriocin 84 (renamed agrocin 84 by Engler *et al.*, 1975) was produced on tomato plants inoculated with strain 84. They observed, as well, a correlation between resistance to agrocin 84 and lack of pathogenicity. This found corroboration in the work by Roberts and Kerr (1974) who observed that conversion of a non-pathogen to a pathogen, via conjugation in planta, was parallelled by a change from agrocin 84 resistant to agrocin 84 sensitive phenotype. Engler *et al.* (1975), using plasmid curing methods, showed that this was so because sensitivity to agrocin 84 was encoded in the Ti-plasmid (which had been shown to be responsible for the pathogeneicity of an *A. tumefaciens* strain ,C58 by Van Larebeke *et al.*, 1974).

1.4.2 SENSITIVITY TO AGROCIN 84

Engler *et al.* (1975) demonstrated a correlation between sensitivity to agrocin 84 and oncogenicity by curing *A. tumefaciens* strain C58 of its nopaline-type Ti-plasmid and showing that the cured isolates were non-pathogenic and resistant to agrocin 84. They also noted, however, that some oncogenic strains bearing Ti-plasmids were resistant to agrocin 84. This observation remained controversial until contributing information from Sule and Kado (1980), Holsters *et al.* (1980) and Engler *et al.* (1981) solved the dilemma. Their work showed that (i) agrocin 84 uptake and virulence are conferred in separate loci of the pTi-plasmid (Sule and Kado, 1980) and that (ii) sensitivity to agrocin 84 is encoded in a region unique to the nopaline pTiC58 plasmid (Holsters *et al.*, 1980; Engler *et al.*, 1981). The agrocin sensitivity region was detected by Holsters *et al.* (1980), through the generation of transposon-induced deletions maps, in the 87 Md region of pTiC58. This region was found to be absent from octopine plasmids (Engler *et al.*, 1981) by DNA homology studies. This explained why strains harboring an octopine plasmid are naturally resistant to agrocin 84.

Murphy and Roberts (1979) were the first authors to suggest that the basis for agrocin sensitivity was a highly specific active uptake system. They were able to show that the agrocin sensitivity region codes for a hydrophobic protein which is found in the periplasmic space, where it binds ^{32}P agrocin 84 reversibly. They suggested that this protein is a permease. This, of course, generated a new dilemma. Why would a bacterium that had evolved a sophisticated mechanism to provide itself with an ecological niche destroy itself by having an uptake system for a bacteriocin?. It was postulated that agrocin 84 is an illegitimate substrate that has "pirated" an existing uptake system. Consequently the search for legitimate substrates that would support this hypothesis begun. Ellis and Murphy (1981) detected a new class of opines which interacts with the uptake of agrocin 84 and postulated them to be the legitimate substrate. These opines, phosphorilated sugars, were called agrocinopines.

The ecological advantage that *A. radiobacter* 84 finds in producing agrocin 84 is explained by its capacity to catabolise nopaline. *A. radiobacter* 84 has three plasmids (Merlo and M ster, 1977); a large cryptic plasmid, a small (47.7 kb) plasmid encoding agrocin, pAgK84 (Slota and Farrand, 1982) and a 200 kb plasmid, pAgK84b encoding nopaline catabolism and carrying genes for the mobilisation of pAgK84 (Ellis *et al.*, 1979). Therefore *A. radiobacter* 84 competes with pathogens for nopaline and in addition kills such pathogens by the production of agrocin 84.

Although from the preceding discussion it should follow that all *A. tumefaciens* harboring nopaline-type Ti-plasmids are sensitive to agrocin 84, this is is not the case. Perry and Kado (1982) found that all the biotype 3 strains they tested, regardless of whether they possessed nopaline Ti-plasmids were resistant *in vivo* to agrocin 84.

1.4.3 PRODUCTION OF AGROCIN 84

The production of agrocin 84 is controlled by a transmissible plasmid, pAgK84, which also codes for immunity to agrocin 84 (Ellis *et al.*, 1979). Cooksey and Moore (1981) cured strain 84 of its pAgK84 plasmid with concomitant loss of agrocin 84 production. Transfer of pAgK84 by mobilisation to other *Agrobacterium* strains not producing agrocin 84 results in strains which have acquired the capacity to produce agrocin 84 (Ellis *et al.*, 1979). Plasmid pAgK84 was genetically isolated (free from any opine-catabolic plasmid) by Slota and Farrand (1982) by mobilising it into a Ti-plasmid free strain and subsequently curing the mobilizing element. Hybridisation analysis show that pAgK84 bears no detectable homology to octopine or nopaline-type *Agrobactrium* plasmids (Slota and Farrand, 1982). Farrand *et al.* (1985) have found tha pAgK84 is a conjugal plasmid. They used the kanamycin-resistance transposon Tn5 as a marker in conjugations with other *Agrobacterium* strains as well as with *Rhizobium meliloti* It has been shown that a 20 kb fragment of pAgK84 is responsible for the production of agrocin 84 (Farrand *et al.*, 1985; Kerr and Tate, 1984).

Thomson (1985) has pointed out that there are at least two potential mechanisms of breakdown in biological control of crown gall by *A. radiobacter*84. One mechanism involves the acquisition of pAgK84 by a pathogeneic strain, which would retain its pathogenicity, produce agrocin an be immune to it. This is not unlikely, Thomson (1985) points out, because in addition to coding for nopaline catabolism, pAt84b carries genes for the mobilisation of pAgK84. The transfer genes, normally repressed, are inducible by nopaline or agrocinopine A (Ellis *et al.*, 1982). Morover, pAt84b was shown to be self transmissible at a very low rate (Slota and Farrand, 1982). Mobilization of pAtK84 would occur, however, only under conditions of ineffective biological control. Nopaline synthesized in galls would then be available, with the concomitant derepression of the

mobilisation functions (Ellis and Kerr, 1979; Panagopoulos *et al.*, 1979). Kerr and Tate (1984) in order to prevent transfer of pAgK84 to pathogens, are developing a strain carrying a transfer and mobilisation deficient pAgK84 derivative.

The second mechanism potentially able to break down biological control is through the acquisition by strain 84 of a Ti-plasmid. This is possible because pTiC58 carries genes for self transmission. Such genes are normally repressed but are inducible by nopaline. It follows that under conditions of inefficient control nopaline produced by crown galls could facilitate transfer of a Ti-plasmid to strain 84. To prevent this, Hendson and Thomson (1985) transferred an agrocin-encoding plasmid to *Rhizobium meliloti*, (a bacterium unlikely to receive or maintain a Ti-plasmid). Hooikas *et al.* (1977) had suggested that the rhizosphere bacteria in general would be suitable candidates for the control of root diseases. As the donor strain Hendson and Thomson (1985) used *A. tumefaciens* 396, a fast growing strain (Hendson *et al.*, 1983) producing an agrocin with the same host range than strain 84 (Engler *et al.*, 1975). Plasmid pAg396 was mobilized with RP4 to *R. meliloti* RF10 where it was stably maintained for at least 36 generations. *R. meliloti* RF10 (pAg396) produced as much agrocin as the parental strain 396 and inhibited gall development by strain C58 on *Datura* plants in glasshouse trials. Field trials are lacking.

1.4.4 STRUCTURE AND MODE OF ACTION OF AGROCIN 84

Agrocin 84 was isolated first from solid medium (Heip *et al.*, 1975) and described as a small peptide (Mac Cardell and Pootjes, 1976), but further studies would indicate that this was no correct. Roberts and Kerr (1977) isolated agrocin 84 from liquid medium and concluded, from structural studies, that it was a fraudulent member of the up until then unreported 6-N-phosphoramidate series of adenine nucleotides. Other studies (Tate

et al., 1979; Thompson *et al.*, 1979) contributed to establish that agrocin 84 is a disubstituted fraudulent nucleotide with a 9-(3'-deoxy-β-D-threopentofuranosyl)-adenine nucleoside core. Once that the structure of agrocin 84 had been clearly defined structure-function studies followed in order to explain the remarkable selectivity of the molecule for bacteria harboring nopaline Ti-plasmids and in what manner it inhibited the growth of target bacteria. Murphy *et al.* (1981) produced, by physicochemical means, derivatives of ^{32}P agrocin 84 lacking either the substituents at N^6 (D-glucofuranosyloxyphosphoramidate) or at C-5 (D-*threo*-2-3-dihydroxy 4 methylpentamide) and studied their incorporation and toxicity. Toxicity was found to be due to the presence of the substituent at C-5' which, in contrast to the substituent at N^6, was incorporated passibly at a barely measurable rate. Data of Murphy *et al.* (1981) indicated the absolute requirement of the N^6-D-glucofuranosyloxyphosphoramidate substituent in the binding-recognition step of agrocin 84 transport. Since the high bacteriocin-like specificity of agrocin 84 is due to its uptake into strains containing a Ti-plasmid-coded protein which recognizes and binds to complementary sites on the agrocin 84 molecule (Murphy *et al.*, 1979), it follows that the N^6-substituent is the moiety of the agrocin molecule detected by the specific periplasmic permease.

The mode of action of agrocin 84 is not completely clear. From a structural point of view, the fraudulent nucleoside core could interfere with any of a large number of steps involving adenine nucleotide intermediates. DNA synthesis has been shown to be affected by agrocin 84 but without causing DNA degradation (Ellis, 1978). This is quite expected since, as Kerr and Tate (1979) pointed out, the lower face of the β-D-3-deoxyarabinofuranosyl group of the agrocin 84 molecule is structurally equivalent to a dideoxynucleoside, an ideal DNA chain terminator. Although this is likely the actual mechanism of inhibition, other factors must be involved since Murphy *et al.* (1979) demonstrated that uptake of

a molecule, derived from agrocin 84, containing a fraudulent nucleoside is not sufficient for growth inhibition of the bacteria. The presence of a 5'-phosphoryl substituted nucleoside was shown to be a requisite for toxicity (Murphy *et al.*, 1979), therefore the toxicity of the agrocin 84 molecule may depend on a 5-phosphoramidate bond. It is not clear if such requirement is for toxicity *per se* or it is because the molecule has to be transformed into an active form (Murphy *et al.* 1981).

1.4.5 RECENT WORK ON NEW AGROCINS

Biological control of crown gall by agrocin 84 is limited to its very specific host range. Only strains of *A. tumefaciens* harboring nopaline-type Ti-plasmids are potentially sensitive to it, but among these are some found to be resistant either *in vitro* or *in vivo* (Kerr and Htay, 1974: Kerr and Panagopoulos, 1977; Moore and Warren, 1979). Strain 84 has failed to control some pathogenic European strains tested (Moore and Warren, 1979). It also failed to control apple and rose plants in parts of America. In Greece no biotype 3 strain isolated from grapevine galls was biologically controlled by strain 84. Kerr and Panagopoulos (1977) isolated new agrocin producing strains from soils, with the hope of finding one that would be able to kill agrocin 84 resistant strains, but none of the strains were effective for biological control.

In South African Soils crown gall is a common and important disease of deciduous fruit trees, grapevine and some ornamentals (Du Plessis *et al.*, 1984), all three biotypes are present (Duplessis *et al.*, 1984; Kerr and Panagopuolos, 1977;). Hendson *et al.* (1983) state that not all strains of *A. tumefaciens* found in South Africa are sensitive to agrocin 84. Therefore, Hendson *et al.* (1983) screened local isolates of *A. tumefaciens* strains for the production of new agrocins potentially useful in crown gall control. They isolated a strain of *A. tumefaciens* ,D286, from a gall in an *Eucaliptus* tree. Strain D286 produced an agrocin with

a broader host range than that of agrocin 84. The strain spontaneously lost its pathogenicity, which added to its potential as an alternative to strain 84 for biological control of crown gall. Hendson *et al*. (1983) showed that sensitivity to agrocin D286 was plasmid encoded and that sensitivity mapped in the region from 11 to 88 Md of four deletion mutants of the nopaline Ti-plasmid pTiC58.

Characterization of agrocin D286 partially purified carried out by Hendson *et al*. (1983) showed that it was resistant to DNAse, RNAse and protease. The UV absorption spectrum of Agrocin D286, with a maximum at 264 nm and a shoulder at 270nm resembled that of the adenine-N^6-phosphoramidate (Tate and Kerr, 1977). This indicates that the possible nature of agrocin D286 is a fraudulent nucleotide as well.

A. tumefaciens D286 carries a 300 Md plasmid, probably a cryptic plasmid. It also bears a second plasmid, slightly smaller than the 132 Md pTiC58. It has not been established, yet, whether the smaller plasmid encodes agrocin production because attempts to mobilise it, cure it, or mutate it have been unsuccessful. Recent evidence, obtained in the CSIR LMCB (Johannesburg), using transposon mutagenesis indicate that chromosomal sequences are involved in the production of agrocin D286.

Recently, studies where the susceptibility of sixty five strains and isolates of *A. tumefaciens*, representing each of the known biotypes, to *A. radiobacter* 84 and *A. tumefaciens* D286 was tested (Van Zyl *et al*., 1986) have been carried out. Results suggest that under field conditions strain D286 or a combination of strains D286 and 84 may occasionally have an advantage over strain 84 alone in combating biotype 1 and 2 pathogenic strains but overall results indicated that strain 84 was able to control tumor formation better than strain D286. Both strains, alone or in combination, failed to control biotype 3 strains. Van Zyl *et al*. (1986) also suggested that their data supports the blockage-of-infection-site hy-

pothesis (Lippincot and Lippincot, 1969; Whatley *et al.*, 1976) as agrocin-susceptible and resistant strains generally responded alike in *in vitro* experiments. Therefore, they pointed out, this is an indication that agrocin involvement was not the major cause of of tumor inhibition by the agrocinogenic strains.

Up to date there is no effective biocontrol agent of crown gall on grapevines, regardless of the catabolic nature of their Ti-plasmids. (Most biotype strains carry octopine Ti-plasmids and some can catabolise both octopine and nopaline (Panagopoulos *et al.*, 1978). In search for an *A. tumefaciens* strain able to control crown gall in grapevine Staphorst *et al.* (1985) isolated, from gall tissue of grapevines, a number of both pathogenic and non pathogenic biotype 3 strains that showed strong *in vitro* agrocinogenic activity against pathogenic biotype 3 isolates. Some of the strains reduced crown gall formation on grapevines in glasshouse experiments. Webster *et al.* (1986) screened isolates from crown galls in search for agrocines active against grapevine isolates. They reported that a pathogenic, biotype 2 strain, J73, isolated from an infected *Prunus salicinia* tree, inhibited grapevine pathogens both *in vitro* and *in vivo*. Experiments in vivo were possible because, although a pathogen, strain J73 does not form crown gall on grapevines. Field trials will soon follow, since strain J73 has apparently been cured of its Ti-plasmid (Webster, personal communication). An interesting feature of agrocin J73 is that sensitivity to it is not encoded by a Ti-plasmid (Webster *et al.*, 1986), since pathogens cured of their Ti-plasmid were still inhibited.

2 INTRODUCTION

Crown gall is caused by strains of *A. tumefaciens* which harbor a tumor inducing (Ti) plasmid (Zaener *et al.*, 1974). During infection part of the Ti-plasmid, the T-region, is transferred from the bacterium to the plant cells where it integrates into the plant nuclear DNA (Chilton *et al.*, 1980) and as a consequence the plant cells undergo cancerous growth to form an undifferentiated gall. The T-DNA also directs the synthesis of opines (Petit *et al.*, 1978) of which there are a number of types including nopaline, octopine, agropine and the recently discovered succinamopine (Chilton *et al.*, 1984). In addition, genes carried on the Ti- plasmid enable its host bacterium to catabolize the relevant opine and, at least in part, determine the bacterium's host range (Thomashow *et al.*, 1980; Buchholz and Thomashow, 1984; Hoekema *et al.*, 1984).

Crown gall has been prevented in some species of plants by a novel means of biological control. The non-pathogenic *A. radiobacter* strain 84 was found to inhibit gall formation in peach seedlings when inoculated together with a pathogen strain (New and Kerr, 1972). The mechanism of control was reported to be due to the production by strain 84 of a bacteriocin-like antibiotic (Kerr and Htay, 1974). The bacteriocin-like antibiotic was later named agrocin 84 by Engler *et al.* (1975) who showed that sensitivity to it in *A. tumefaciens* strain C58 was encoded by the Ti-plasmid responsible for its pathogenicity. Sensitivity to agrocin 84 was found to be encoded in a region unique to the nopaline Ti-plasmid pTiC58 Engler *et al.*, 1981), therefore strains harboring an octopine plasmid are naturally resistant to agrocin 84. In addition, Perry and Kado (1982) found that the resistance *in vivo* to agrocin 84 in all biotype 3 strains they tested was independent of whether they possessed nopaline Ti-plasmids. This limits the use of agrocin 84-producing strains for biological control even further.

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