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Review

Crown Gall Disease and Hairy Root Disease[1]

A Sledgehammer and a Tackhammer

Stanton B. Gelvin

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

ABSTRACT

The neoplastic diseases crown gall and hairy root are incited by the phytopathogenic bacteria *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, respectively. Although the molecular mechanism of T-DNA transfer to the plant most likely is the same for both species, the physiological basis of tumorigenesis is fundamentally different. Crown gall tumors result from the overproduction of the phytohormones auxin and cytokinin specified by *A. tumefaciens* T-DNA genes. Although the T-DNA of some Ri-plasmids of *A. rhizogenes* contains auxin biosynthetic genes, these loci are not always necessary for hairy root formation. Recent experiments suggest that hairy root tumors result from the increased sensitivity of transformed cells to endogenous auxin levels. An understanding of hairy root tumorigenesis will likely result in an increased knowledge of plant developmental processes.

Crown gall disease and hairy root disease are neoplastic growths on plants incited by virulent strains of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, respectively. Depending upon the strain of bacterium used, the morphology of crown gall tumors is typified by either the production of amorphous, unorganized callus or by teratomas containing aberrantly organized stem and leaf-like structures. In addition, the host plant species and even the position of inoculation on the plant can in part determine the tumor morphology. Hairy root tumors are distinguished by a massive proliferation of roots, frequently harboring numerous adventitious root hairs, emanating from the site of infection (see Fig. 1 for examples of crown gall and hairy root tumors). Whereas crown gall tumors rarely revert to tissue that is capable of regenerating plants, hairy root tumors of numerous plant species can spontaneously regenerate plants. These plants differ from normal plants in several regards, however: they frequently show alterations in leaf pigmentation and morphology, internode length, root geotropism, flower morphology, and plant generation time.

The determinants of both crown gall and hairy root disease are large plasmids harbored by the virulent bacterium. In the case of *A. tumefaciens*, these plasmids are termed Ti-(tumor inducing) plasmids. Virulent *A. rhizogenes* strains harbor Ri-(root inducing) plasmids. There are numerous classes of both Ti- and Ri-plasmids, but they share certain characteristics. They are large (200 to greater than 800 kbp) and contain two regions necessary for tumorigenesis. These include the T-(transferred) DNA region, that is destined to be transferred to the plant cell, and the *vir* (virulence) region.

The T-DNA is delimited by 24 bp directly repeated DNA sequences that are very similar between Ti- and Ri-plasmids (18). Many Ti- and Ri-plasmids contain two T-DNA regions that can be independently transferred to the plant. After transfer, the T-DNA integrates into the plant nuclear DNA where its genes are transcribed by RNA polymerase II. The T-DNA contains genes that specify the oncogenic phenotype. In addition, the T-DNA harbors genes that direct the production and secretion of low mol wt, tumor-specific compounds termed opines. These opines can be utilized by the inciting *Agrobacterium* strain as the sole source of carbon and, in some instances, nitrogen. In addition, some opines can induce the conjugal transfer of Ti- or Ri-plasmids between bacterial cells.

The *vir* region of the Ti- and Ri-plasmids contains numerous genes involved both in the processing of the T-DNA from the larger plasmids and in its transfer from the bacterium to the plant cell. *vir* region genes from most Ti- and Ri-plasmids are very similar, and the *vir* genes from one *Agrobacterium* species can mediate the transfer of the T-DNA from another species when placed in the heterologous bacterial cell (7).

Because of the similarity of the structure of Ti- and Ri-plasmids, as well as the similarity between the molecular mechanisms by which the T-DNA is processed and transferred to plant cells from Ti- and Ri-plasmids, many scientists initially suspected that the physiological basis for crown gall disease and hairy root disease would be the same. Subsequent investigations showed, however, that these initial views were too simplistic. Whereas we now know much about the physiological basis for crown gall disease, recent experiments suggest that the mechanism of oncogenesis in hairy root disease differs significantly from its crown gall counterpart. A thorough understanding of hairy root tumorigenesis may lead scientists to an increased understanding of normal plant development.

PHYSIOLOGICAL BASIS FOR CROWN GALL TUMORIGENESIS

The physiological basis for crown gall tumorigenesis was first suggested by experiments in which mutations were intro-

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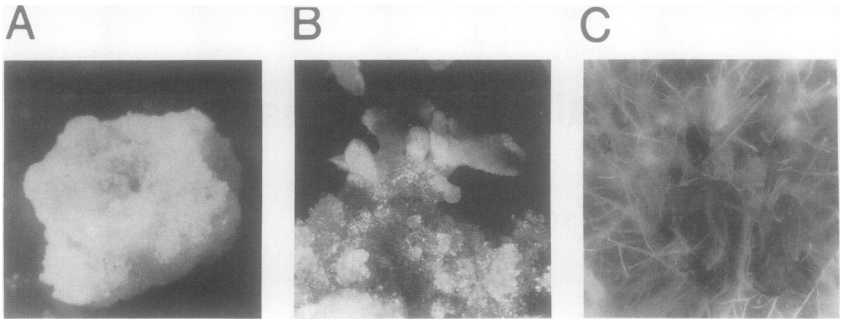

Figure 1. Tobacco tumors in axenic tissue culture. A, An unorganized crown gall tumor incited by *A. tumefaciens*; B, a shooty crown gall teratoma incited by *A. tumefaciens*; C, a hairy root tumor incited by *A. rhizogenes*. Note the spontaneous regeneration of a plant from the hairy root tissue.

duced into the T-DNA, and the resulting plasmid used to induce tumors on a number of different plant species. Some of these mutations led to the production of altered tumor morphologies on a number of these hosts. For example, a wild-type *Agrobacterium* strain that would normally induce an unorganized callus tumor would now produce tumors that appeared much like either shooty teratomas or hairy root tumors, depending upon the position of the mutation in the T-DNA (6). The addition of auxin to the wound tissue at the time of infection would alter a mutant shoot-inducing *Agrobacterium* strain to one which would once again generate unorganized tumor callus (8). Such experiments suggested that the physiological basis for crown gall disease depended either upon the novel production by the tumors of plant growth regulating substances, such as auxin and cytokinin, or by the alteration of the plant's control of or response to phytohormone production. Garfinkel *et al.* (6) produced the first detailed map of the T-DNA of an octopine-type Ti-plasmid using insertion mutagenesis. Their genetic studies suggested that mutations in two genes (tumor morphology shooty 1 and 2, or *tms1* and *tms2*) were responsible for altering this *Agrobacterium* strain from one that normally generated unorganized tumors to one that now initiated shooty teratomas. Another mutation, at the *tmr* (tumor morphology rooty) locus, resulted in the production of tumors from which proliferated roots. The results of these genetic experiments were reminescent of the results obtained previously by Skoog and Miller (17). In a classic series of experiments, these investigators altered the morphology of nontransformed tobacco callus by incubating the tissue on media containing various ratios of auxin and cytokinin. The results of the experiments of Garfinkel *et al.* (6) also suggested that the mutations in the T-DNA resulted in an altered phytohormone balance: *tms* mutations led to tumors that had a high cytokinin to auxin ratio, whereas *tmr* mutations resulted in tumors that had a high auxin to cytokinin ratio. Unorganized tumors would have an intermediate ratio of these two growth regulators. Chemical analysis of the auxin and cytokinin levels in wild-type, *tms*, and *tmr* tumors confirmed this hypothesis (2).

The biochemical basis for phytohormone overproduction by crown gall tumors was subsequently determined by a number of laboratories. Experiments in *A. tumefaciens*, *Escherichia coli*, and in plants indicated that the *tms* loci encoded enzymes involved in a two step pathway for the production of auxin from tryptophan: The *tms1* gene encodes the enzyme tryptophan monooxygenase that converts tryptophan to the intermediate compound indole-3-acetamide (22, 23). This intermediate is subsequently converted to the auxin IAA by the product of the *tms2* gene, an amidohydrolase (14, 21). Although this two step pathway for auxin production differs from the three step pathway (via, *e.g.* tryptamine) utilized by plants, it is identical to the method by which another tumorigenic bacterium, *Pseudomonas syringae* pv *savastanoi* (*Pseudomonas savastanoi*), synthesizes auxin. *P. savastanoi* incites the disease olive knot on olive and oleander plants. The disease is caused by the production and secretion of the phytohormones$_I$ auxin and cytokinin$_I$ by the bacterium into the plant. *P. savastanoi* harbors genes encoding the enzymes tryptophan monooxygenase and indole acetamide hydrolase. In some strains, these genes are found on a plasmid. The *Pseudomonas* IAA biosynthetic genes share considerable nucleic acid and amino acid sequence homologies with their *Agrobacterium* counterparts (25). Neither, however, shows significant homology to the DNA of most plant species investigated.

The *tmr* gene of the *A. tumefaciens* T-DNA encodes an isopentenyl transferase that transfers the isoprenoid side chain from dimethyl-allylpyrophosphate to 5′ AMP, yielding the cytokinin *iso*-pentenyladenosine 5′-P (1, 3). Such a gene (*ptz*) has also been found on a *P. savastanoi* plasmid. The two genes show considerable nucleic acid and amino acid sequence homologies, as does a third cytokinin biosynthetic gene, *tzs*, found near the *vir* region of nopaline-type Ti-plasmids (11). Thus, both *A. tumefaciens* and *P. savastanoi* generate tumors by directing the overproduction of auxins (via a novel pathway) and cytokinins in plant tissues. The difference between the tumorigenic mechanism employed by these two bacteria lies in the site of phytohormone production: olive knot disease depends upon the continuous presence of the pathogenic bacterium that is responsible for auxin and cytokinin production. For crown gall tumorigenesis, the continuous presence of the bacterium need not exist because the bacterium has (with the exception of the tzs gene) transferred the phytohormone biosynthetic genes to the plant.

PHYSIOLOGICAL BASIS FOR HAIRY ROOT DISEASE

Despite the similarities between the *vir* genes and the T-DNA borders of Ti- and Ri-plasmids, which most likely reflect identical mechanisms of T-DNA transfer from *A. tumefaciens* and *A. rhizogenes* to plant cells, we now know that the physiological bases for crown gall and hairy root disease differ

significantly. To some extent, this may have been predictable considering the regenerative capabilities of tumor tissue incited by these two bacteria. Crown gall tumors rarely regenerate plants capable of rooting. When plants that have regenerated from these tumors were analyzed, they had either deleted the majority of T-DNA sequences or had inactivated T-DNA gene expression via methylation of T-DNA sequences. Hairy root tumors from a number of plant species, however, spontaneously regenerate plants, albeit with altered morphology, that still contain and express T-DNA genes (5, 20).

A number of different groups of Ri-plasmids have been characterized. The classification of these plasmids has to a large extent depended upon the types of opines (mannopine, agropine, cucumopine) that the T-DNA of these plasmids direct the infected plant to synthesize. The most frequently investigated *A. rhizogenes* strains harbor the agropine-type Ri-plasmids pRiA4 or pRi1855, or the mannopine-type Ri-plasmid pRi8196. The agropine-type Ri-plasmids harbor two T-DNAs, T_L (T-DNA left) and T_R (T-DNA right). The mannopine-type and cucumopine-type Ri-plasmids contain only one T-DNA that shares considerable DNA sequence homology with T_L of the agropine-type plasmids. DNA hybridization analysis of the T_R region of the agropine-type Ri-plasmids indicates that it is homologous to the *tms1* and *tms2* genes of the *A. tumefaciens* Ti-plasmids. One may therefore surmise that the transfer of these auxin biosynthetic genes into plant cells would account for the hairy root phenotype. However, the T-DNA of mannopine-type Ri-plasmids does not hybridize to the *A. tumefaciens tms1* or *tms2* genes (10), despite the fact that mannopine-type *A. rhizogenes* strains incite hairy root tumors on a number of plants. In addition, the molecular analysis of DNA from a number of hairy root tumors generated by infection of plants with agropine-type *A. rhizogenes* strains indicates that the T_R DNA (harboring the genes homologous to the Ti-plasmid auxin biosynthetic genes) need not be present at detectable levels to obtain the hairy root phenotype (9). Thus, there appears to exist two mechanisms of hairy root tumorigenesis: one (although weak, see below) depends upon auxin overproduction directed by the T_R T-DNA of certain *A. rhizogenes* strains, but the other is apparently independent of the transfer and expression of genes directing the biosynthesis of auxin.

The first intensive genetic examination of *A. rhizogenes* T-DNA gene function was conducted by White *et al.* (24). These investigators generated a large number of transposon insertion and small deletion mutations in the agropine-type Ri-plasmid pRiA4. Mutations in the T_L region were especially interesting. When *A. rhizogenes* strains harboring these mutations were inoculated onto the leaves of *Kalanchoe diagremontiana*, a number of phenotypes differing from the wild-type response were noted. Four genetic loci (termed *rolA, rolB, rolC,* and *rolD*) were defined according to the tumor morphology observed: *rolA* mutants generated roots that were very straight (as opposed to the curled roots obtained using the wild-type T-DNA). *rolB* mutants were avirulent. Tumors incited with *rolC* mutants showed attenuated root growth (although callus growth was normal), and *rolD* mutants showed the initiation, but subsequent retardation, of root growth. Thus, the importance of specific genes in the T_L region of agropine-type Ri-plasmids was defined. Subsequent DNA sequence and transcript analysis of this region indicated that the *rol* genes corresponded to specific open reading frames (18).

The functions of the pRi T_L genes in hairy root tumorigenesis were suggested by experiments in which carrot root disks were inoculated with various *A. tumefaciens* and *A. rhizogenes* strains (12). Most *A. tumefaciens* and agropine-type *A. rhizogenes* strains could incite tumors when inoculated either on the apical (side facing the root tip) or the basal (side facing the shoot) surface of the disk. *A. tumefaciens tms* mutations, however, could only efficiently incite tumors when inoculated onto the apical surface, suggesting that polar auxin transport to this surface could relieve the deficiency caused by the mutations in the auxin biosynthetic genes. Interestingly, a similar phenomenon was observed with mannopine- type *A. rhizogenes* strains: hairy root tumors could only form following the inoculation of the bacteria on the apical surface of the carrot disk, although mannopine was produced by cells inoculated on the basal surface, indicating that T-DNA transfer to these cells had occurred. Virulence on the basal surface could be restored by adding naphthalene acetic acid, an auxin, at the time of inoculation. Incubation of carrot disks with auxin alone (with or without cytokinin) did not result in hairy root formation (4). Cardarelli *et al.* (4) also showed that the addition of the T_R region of an agropine-type Ri-plasmid to the mannopine-type *A. rhizogenes* strain could result in 'nonpolar' infection properties: such strains could now incite hairy root tumors on either surface of carrot disks. Mutation of the T_R genes homologous to the *tms* genes resulted in an agropine-type strain that could now generate hairy roots only on the apical surface of carrot disks. In addition, the presence of the T_R region alone from an agropine-type *A. rhizogenes* strain resulted in a bacterium that showed only weak root formation on carrot disks, indicating that the auxin biosynthetic genes harbored on the T_R DNA performed a relatively minor role in hairy root formation. Finally, the authors showed that when a mannopine-type *A. rhizogenes* strain was killed following infection of the basal surface of a carrot disk, hairy roots could form if an auxin were subsequently provided to the plant cells.

Taken together, these results suggested that the T_R auxin genes were at best weak oncogenes; they could only function to generate hairy roots efficiently when in the presence of the T_L *rol* genes. In addition, the T_L *rol* genes of the agropine-type Ri-plasmids, and the analogous genes harbored by the mannopine-type Ri-plasmids, functioned by sensitizing the plant cells to auxin. That this latter hypothesis was most likely correct was further demonstrated by Shen *et al.* (15). These authors investigated various physiological properties of protoplasts derived from root tips of *Lotus corniculatus* that were either nontransformed or transformed by *A. rhizogenes.* Measurements of root tip elongation rates, proton excretion, and the transmembrane electrical potential indicated that hairy root transformed cells were 100 to 1000-fold more sensitive to the effects of auxin than were nontransformed cells. The authors hypothesized that this modified response to auxin by the transformed cells was an early cellular event,

possibly involving the reception or transduction of the hormone signal.

A. RHIZOGENES rol GENES AND PLANT DEVELOPMENT

Although hairy root tumors incited on a number of plant species could regenerate plants, these regenerants frequently differed from their normal counterparts in a number of parameters: they often had wrinkled leaves, altered internode lengths, pleigeotropic roots, and reduced apical dominance and fertility. In addition, biennial species frequently became annuals (20). The participation of each of the *A. rhizogenes rol* genes in these phenomena was assessed by a number of laboratories.

Leaf sections of plants regenerated from *Nicotiana tabacum* hairy root tumors form roots at the wound margins when cultured on hormone-free medium. Non-transformed leaf tissue can elicit the same response when grown in the presence of auxin. Spano *et al.* (19) showed that the ability of hairy root regenerants to form roots in culture was independent of the T_R region of the Ri-plasmid that contained the auxin genes. Such an effect could also be obtained when only the genes *rolA, rolB,* and *rolC* (open reading frames 10, 11, and 12 of Slightom *et al.* [18]) were used to transform tobacco cells. The *rolB* locus alone was not sufficient to elicit the full hairy root phenotype. Spano *et al.* (19) additionally showed that the auxin concentration in the leaves of hairy root plants was at most twice that found in the leaves of normal plants. This increase in auxin concentration was not enough to cause rooting of leaf sections in tissue culture. Thus, the hairy root phenotype could not merely be attributed to a large increase in the auxin concentration of plant tissues. Sinkar *et al.* (16) also showed that the T_L region of an agropine-type Ri-plasmid was sufficient to elicit the full range of symptoms in regenerated hairy root tobacco plants. Individual mutations in each of the *rol* genes were made, and tobacco plants harboring the mutant Ri T-DNA regenerated. Only mutations in the gene *rolA* resulted in the loss of the wrinkled leaf phenotype. When the *rolA* locus alone was transferred to tobacco plants, the wrinkled leaf phenotype among the regenerants was observed. However, other hairy root traits were not present in these latter plants. These results indicated that the *rolA* gene was responsible for the wrinkled leaf phenotype, but that other *rol* genes contributed in an important way to the full hairy root phenotype.

A comprehensive study of the effect of each of the *rol* genes on the hairy root phenotype was conducted by Schmulling *et al.* (13). As suggested by the previous studies, these authors showed that tobacco plants containing either the *rolA* or the *rolB* plus *rolC* genes did not show the full hairy root phenotype. When plants containing these combinations of *rol* genes were crossed, however, 25% of the progeny displayed full hairy root symptoms. Plants containing *rolC* alone showed altered leaf morphology, increased branching, and reduced flower size and fertility. Plants harboring *rolB* alone had an increased stigma and flower size, adventitious roots emanating from the stem, and displayed heterostyly. The fertility was near normal, however. *rolA* plants showed wrinkled leaves and larger flowers. In each of these situations, the morphologies correlated with the production of *rol* gene transcripts. However, great variability existed among each class of transformed plant, possibly caused by differing levels of expression of the *rol* genes. These genes were, therefore, subsequently placed under the regulation of the strong Cauliflower Mosaic Virus (CaMV) 35S promoter.

Regenerated plants expressing the *rolC* gene to a high level were dwarf and bushy, displaying greatly decreased internode lengths. The plants had small leaves and flowers and were generally sterile. Overexpression of the *rolB* locus resulted in callus that was necrotic and recalcitrant to shoot production. Those plants that were regenerated (perhaps because of the lower expression of the *rolB* gene due to integration position effects) showed heterostyly and leaves with rounded edges that died early. Thus, the effects of the *rolB* and *rolC* genes seemed to counteract each other: the overexpression of the *rolC* locus led to a juvenilization (a cytokinin-like effect), although root proliferation was also stimulated. The *rolB* gene product resulted in early necrosis (an auxin-like effect). Clearly, the interactive expression of all of the *rol* genes at low levels (under the control of their own promoters) was necessary to elicit the complete hairy root phenotype.

Although the symptoms caused by the *rol* genes functioning independently or synergistically are complex, the physiological response of the plant to these gene products typifies various processes in normal plant development. The elucidation of the biochemical events directed by the *rol* genes may offer further insights into plant developmental biology.

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