

Fig. 6. Comparison of protein from tubers and starch-containing petioles by immunoelectrophoresis. Protein from mature underground tubers, selection 77-5-1 (lane 1) and from petioles of stem cuttings with the axillary buds removed which had been grown under short photoperiod for 28 days (lane 3) were separated by electrophoresis and allowed to react with antibody to total soluble tuber protein (trough 2).

at the protein level by rocket immunoelectrophoresis or ELISA and also can be studied at the nucleic acid level by *in vitro* translation or by using recombinant DNA technology to isolate and characterize the mRNAs and genes. Further research is needed to examine the effect of photoperiod, hormones, heat stress, and other stimuli on these proteins and to compare their expression in potato cultivars which differ in traits such as photoperiod responsiveness, starch and protein production, and heat stress resistance.

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AGROBACTERIUM TI PLASMIDS AS POTENTIAL VECTORS FOR GENETIC ENGINEERING

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The use of *in vitro* techniques for DNA manipulation allows individual DNA sequences to be purified from their original genetic background. Such cloned sequences have been of great value in elucidating gene structure and organization; however, an understanding of how genes function requires an assay system in which genes carried on the cloned sequences may be expressed. Once such a system is available, it should be possible to study the expression and regulation of individual genes. One solution to this problem is to introduce cloned genes into cells which are able to express those genes; this approach offers the additional possibility of introducing new traits into cells and is potentially of great agricultural, medical, and commercial importance.

Methods have been devised for introducing foreign DNA into bacterial, yeast, and mammalian cells, but there is no equivalent well-defined, reproducible, and efficient means of transforming plant cells. Direct transformation of plant cells with naked DNA has proved to be problematical and consequently, much interest and research effort now is being directed towards the development of a vector capable of introducing foreign genes into plant cells. Ideally, a genetic engineering vector should fulfill the following criteria: (a) wide host range; (b) an efficient route of entry into the plant cell; (c) facilitate the maintenance and expression of foreign genes within the plant cell; (d) possess an easily selectable marker for scoring transformation events (for example, drug resistance); (e) transmit foreign genes to all cells of the host plant (e.g., by regeneration of plants from single transformed cells) and its progeny; and (f) not adversely affect the health of the host plant.

Many of the above requirements are met by the Ti plasmids of *Agrobacterium tumefaciens* (E.F. Sm & Towns.) Conn, which appears to be a naturally occurring genetic engineering vector. *A. tumefaciens* is a soil-bourne bacterium that incites crown gall disease on a wide variety of dicotyledenous plants [see (17) and (19) for recent reviews]. Crown galls are tumorous growths characterized by the production of novel amino-acid derivatives called opines and by the ability of explanted tumors to grow in the absence of exogenous phytohormones. It is known that the causal agent of the disease is the bacterial Ti plasmid (22, 23), part of which (T-DNA) is stably maintained in tumor cells (4, 6, 24), where it is covalently linked to plant cell nuclear DNA (26, 28). Little is known of the

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mechanisms involved in the delivery of the Ti plasmid (or its possible derivative) into the plant cell and the integration of T-DNA into the genome. The T-DNA encodes both opine production and phytohormone independence (5, 7) and has been shown to produce polyadenylated transcripts (1, 8, 11, 12) that are found on plant polysomes (15, 27). Some of these transcripts encode polypeptide products (15, 25), including the particular enzyme responsible for opine biosynthesis (16, 20, 21). Bacterially harbored Ti plasmids encode the functions for opine catabolism (9) allowing the inciting bacterium to use these compounds as a carbon and nitrogen source. This arrangement, whereby the Ti plasmid subverts the functions of the plant cell towards the needs of the bacterium, has been called genetic colonization.

The Ti plasmid, from the viewpoint of the genetic engineer, demonstrates the feasibility of introducing functioning foreign genes into the plant cell. Conceptually, any DNA sequence could be introduced into the plant cell by inserting desired genes into the T-DNA region of the Ti plasmid prior to its introduction into the plant. Such a scheme exploits the natural ability of Ti plasmids to enter plant cells and introduce a defined segment of DNA into the genome. Such engineered genes will be able to function in the plant only if they contain the appropriate regulatory sequences which are recognized by the plant's transcriptional and translational machinery. Work on the introduction of foreign genes into T-DNA and subsequently into plant cells, which was carried out in our laboratory, is described below.

Site-specific engineering of genes into T-DNA

The large size of Ti plasmids (95 to 156 megadaltons) and the consequent lack of unique sites for cleavage with restriction endonuclease enzymes preclude direct insertion of foreign genes (socalled "target DNA") into specific sites within the T-DNA region. Techniques to overcome this hurdle have been developed both in our laboratory (14) and by Schell's group (13). First, small fragments of the T-DNA region are cloned into ColEl replicon-derived plasmids of Escherichia coli, such as pBR322. Next, target DNA and a marker gene encoding a drug resistance are ligated into the middle of the cloned T-DNA fragment. This is then cointegrated with a wide hostrange plasmid capable of replicating in both E. coli and in Agrobacterium (the RK2-derived plasmid pRK290 in our scheme). Finally, the pRK290-target DNA plasmid is introduced into an Agrobacterium strain harboring the wild-type Ti plasmid. Homology between the T-DNA regions flanking the target DNA and the drug resistance marker and the equivalent T-DNA sequences found on the wild-type Ti plasmid permits replacement of the latter with the engineered T-DNA fragment by means of a double homologous recombination. Double crossover events are selected by the introduction into the Agrobacterium of an "eviction plasmid" which is incompatible with the pRK290-target DNA plasmid and carries a second drug resistance marker. Expression of both drug resistances can only occur if a double crossover has moved the original drug resistance marker onto the Ti plasmid. The target DNA will move with the drug marker, hence the net result is an engineered Ti plasmid in which part of the T-DNA has been replaced by an engineered fragment. This methodology is extremely versatile, since it allows any portion of the Ti plasmid to be mutated, whether by addition, replacement, or deletion of DNA. Agrobacterium strains harboring engineered Ti plasmids may be used subsequently to infect plants and to introduce the target DNA into the plant genome. The chief advantage of the Agrobacterium system is that all of the manipulations required to construct the engineered vector are carried out in a prokaryotic environment which is relatively simple and easy to handle.

This methodology has been used to introduce the neomycin phosphotransferase gene from the bacterial transposon Tn5 and the yeast alcohol dehydrogenase gene into tobacco, and the maize zein gene into sunflower. Hybridization of the DNA from these engineered tumors with radiolabeled probes bearing the authentic target genes (Southern blot analysis) confirmed the presence of the target genes in the plant cell covalently linked to nuclear DNA. It appears that foreign genes may be introduced readily into plant cells by these techniques; however, expression of these genes in plant cells does not appear to be quite so straightforward. No RNA transcripts could be detected in any of the above tumor lines (13, T. Matzke, K. Barton, unpublished data), indicating that the regulatory sequences of these particular genes are either unable to function in their new cellular environment or are expressed only at a particular stage of development that has not yet been examined.

One solution to this problem may be the use of chimeric gene constructions in which the coding sequence of the target DNA is coupled to the regulatory sequences of a gene that is known to function in the host plant cells. One class of regulatory sequences that could be used in this manner are those found on the T-DNA itself. Advantages of these sequences are their ability to function in a wide range of host plants, their compatibility with the Ti plasmid, and their availability. This approach is exploited through the 5' regulatory sequences of the T-DNA which have been identified by mapping the RNA transcripts using Northern analysis, defining the ends of the transcripts by S1 nuclease mapping, and by DNA sequencing of the regions encoding the transcripts (1, 2). One of the most abundant transcripts of the T-DNA is encoded by the gene for nopaline synthase. Chimeric genes have been constructed using the 5' regulatory sequences of this gene inserted into the Ti plasmid, using the site-specific method described above and so introduced into the plant. Expression of these genes in plant cells is being studied currently, and the results are awaited with great interest. This strategy may allow the expression of foreign genes that are unable to promote their own expression in plant cells as well as letting any gene-coding region be placed under the control of particular types of regulating promoters. For example, a gene could be placed under the control of regulatory sequences that allow transcription in response to external stimuli or developmental cues.

The regeneration of healthy plants from transformed tissue

The tumorous phenotype of crown gall tissue is not compatible with the production of healthy genetically engineered plants. A way must be found, therefore, to disrupt the oncogenic functions of the T-DNA without upsetting its ability to integrate into, and be maintained in, the plant genome. Garfinkel and co-workers (10) used transposon mutagenesis of the Ti plasmid in a definitive study to reveal 4 genetic loci in the T-DNA which gave rise to "shooty," "rooty," "large," or octopine negative tumor phenotypes. "Shooty" mutants in one particular study regenerated into apparently healthy, whole plants that contained T-DNA in their tissues and were able to pass the T-DNA-encoded traits to their progeny in a Mendelian fashion (18). It appears that insertion at this locus disrupts the functions responsible for regulating cytokinin levels which ordinarily lead to the expression of the tumorous phenotype. The yeast alcohol dehydrogenase gene mentioned previously was introduced into this particular locus and gave rise, via single cell cloning, to regenerated plants which carried the target gene in all tissues and passed it through meiosis to their progeny (K. Barton, A. Binns, and M-D. Chilton, submitted). However, the expression of this gene has not been detected in these plants or their progeny. The progeny did, however, produce nopaline synthase, although expression appeared to be regulated developmentally (K. Barton, personal communication). These studies suggest that T-DNA can be made nononcogenic without affecting its ability to integrate in plant genome DNA or to promote gene expression.

An alternative approach to the regeneration problem is represented by *Agrobacterium rhizogenes*, a bacterium related to *A. tumefaciens*, which caused "hairy root" disease on dicotyledenous plants (3). The mechanism of root proliferation is analogous to that found for crown gall in that a defined segment of a large plasmid (Ri) is integrated into the plant genome. *A. rhizogenes* may be a naturally occurring, disarmed Ti plasmid, since normal carrot plants can be regenerated routinely from the adventitious roots that proliferate at the wound site. These roots may be cultured *in vitro*, passed through a callus stage and taken through embryogenesis by manipulation of the levels of exogenous phytohormones.

Conclusions and prospects

The introduction of foreign genes into plant cells is now a routine, though lengthy, procedure. By analogy with mammalian genes, it seems likely that foreign genes will be expressible in plant cells when the coding region is provided with the correct regulatory signals. Construction of a chimeric gene that is active in tobacco has been reported recently (15th Miami Winter Symposium). Further studies using regulating promoters should provide much valuable information concerning the DNA sequences required for the timing and extent of transcription in plant cells. One consequence of disarming the Ti plasmid is that the tumerous phenotype is removed as a selectible marker for transformation. The recent success in the expression of a drug resistance marker in tobacco cells demonstrates the feasibility of genetic engineering of plant cells and, additionally, provides an alternative means of selection of transformed cells. Hence, we can look forward confidently to the introduction of foreign genes into plant cells that are able to give rise to whole healthy plants.

An unexplored area of research of great importance if this technology is to be of agronomic use is the stability of T-DNA-introduced genes both within growing plants and from parent to progeny. Host range is another area that is of interest since it may be desirable to enlarge or restrict the number of species that are susceptible to engineered Ti plasmids. In conclusion, plant genetic engineering is in its infancy but the promise of only a few years past has been quickly realized and we may look forward to the routine introduction of genes into plant cells. What is less clear is the significance of this approach for plant breeding, since many desirable traits are either governed by multi-gene families (all of which would have to be transferred and expressed) or simply are not available as cloned DNA sequences. Nonetheless, availability of a transformation system will provide an invaluable aid to the plant biologist interested in understanding how genes work.

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