discrete conductances of 100 to 160 pS occur between isolated pairs of lacrimal or embryonic cardiac cells (*19*) and in isolated gap junctions incorporated onto lipid membranes (*20*).

Direct intercellular pathways that are shared by ions and other solutes, and their regulation in an all-or-none fashion, have important consequences for the role of gap junctions in physiological and pathological processes. In this situation all permeant molecules, regardless of size or charge, are similarly affected.

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Transformation of *Arabidopsis thaliana* with *Agrobacterium tumefaciens*

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Transformed *Arabidopsis thaliana* plants have been produced by a modified leaf disk transformation-regeneration method. Leaf pieces from sterilely grown plants were precultured for 2 days and inoculated with an *Agrobacterium tumefaciens* strain containing an avirulent Ti (tumor-inducing) plasmid with a chimeric gene encoding hygromycin resistance. After cocultivation for 2 days, the leaf pieces were placed on a medium that selects for hygromycin resistance. Shoots regenerated within 3 months and were excised, rooted, and transferred to soil. Transformation was confirmed by opine production, hygromycin resistance, and DNA blot hybridization of both primary transformants and progeny. This process for producing transgenic *Arabidopsis* plants should enhance the usefulness of the species for experimental biology.

THE VIRTUES OF *Arabidopsis thaliana* as an experimental model plant for genetic, biochemical, and molecular biological studies have been extensively reviewed (*1*). The useful features of this remarkable plant include the following. It is small, prolific, and easy to grow and has a generation time as short as 5 weeks. Many mutations have been identified, studied, and mapped and are readily available. Its five chromosomes correspond to five linkage groups and contain only about 7×10^7 bp, the smallest genome known in the angiosperms (*2*). One experimentally useful technique that has been needed is a facile method to introduce natural or modified genes into *A. thaliana* to obtain complementation of mutants or to study factors involved in gene expression. By applying the tumor-inducing (Ti) transformation system developed for other dicots, we have developed a simple procedure to obtain transgenic *A. thaliana* plants.

Agrobacterium tumefaciens provides a natural gene-transfer mechanism that can be utilized to transfer a defined DNA sequence into the genome of cells of many dicotyledonous plants (*3*). Transgenic plants can be created in a simple process that involves a useful selectable marker and the capacity of somatic cells to regenerate new meristems, as illustrated by the leaf disk–transformation technique with tobacco, petunia, and tomato (*4*). Adaptation of the leaf disk system to *A. thaliana* required a new selectable marker since direct selection for kanamycin resistance conferred by our pMON200 vector (*5*) was not efficient in this species. The inefficiency in selection was characterized by growth of callus from uninfected control tissue and by poor recovery of genuinely transformed tissue on medium containing kanamycin.

Hygromycin B is an aminocyclitol antibiotic that inhibits protein synthesis in prokaryotic and eukaryotic cells (*6*). A gene from a bacterial resistance (R) factor that encodes a hygromycin phosphotransferase (hph) has been used to construct chimeric genes that act as dominant selectable markers for transformation of yeast, mammalian cells, and plants (*7, 8*). These results led us to construct and test an hph marker for transformation of *Arabidopsis*. The hph coding sequence was inserted into the

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pMON237 expression cassette vector (*9*) to create pMON404 (Fig. 1), which carries a chimeric gene consisting of the cauliflower mosaic virus (CaMV) 19*S* promoter, the hph coding sequence, and the nopaline synthase polyadenylation signals. The hph coding sequence was inserted in the reverse orientation in pMON405, which served as a control in transformation experiments. The pMON404 and pMON405 plasmids were transferred into *A. tumefaciens* strain A208 carrying the avirulent nopaline-type pTiT37-SE plasmid (*10*) by a process analogous to that for the previously described SEV system (*5*).

There are several published procedures for culture and regeneration of *A. thaliana* (*11*). We simplified the callus induction–regeneration regime in that we used one medium (*12*), the same used for petunia and tobacco (*4*).

To produce transgenic plants, we cultured leaf explants from sterile seedlings (*12*) on the callus-regeneration medium for 2 days and then transferred them to 4 or 5 ml of liquid MS medium in a sterile 150- by 15-mm petri dish, taking care to prevent desiccation during handling. In these experiments, no more than four explants were taken from any individual seedling. *Arabidopsis thaliana* var. Columbia has been maintained as a pure self-fertilizing line (*12*) so that seedling variation should be minimal. One or 2 ml of an overnight culture of *A. tumefaciens* (*4, 5*) was added, and the pieces were gently agitated to wet all surfaces and allowed to soak for 1 to 2 minutes. The inoculated leaf sections were then blotted on sterile filter paper to remove the excess liquid and placed on tobacco cell feeder culture plates (*4*). Feeder culture plates were not absolutely necessary for transformation, but they greatly reduced bacterial damage to the leaf pieces and improved overall transformation results. After cocultivation for 2 days, the leaf pieces were transferred to the same medium, without the feeder cells, and containing carbenicillin (Pfizer, 500 μg/ml), 2.5 m*M* arginine, and hygromycin B (Calbiochem-Behring, 10, 20, or 30 μg/ml).

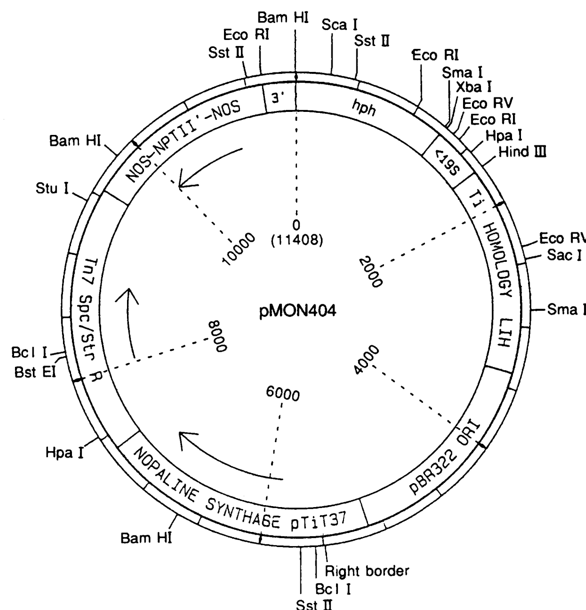

Fig. 1. Structure of pMON404, which contains the chimeric hph gene. Gritz and Davies (*7*) described two plasmids that carry the hph coding sequence and that have Bam HI linkers inserted near the 5′ end (pLG89) or 3′ end (pLG83). A full-length hph coding sequence was assembled by joining the 0.26-kb Bam HI–Eco RI fragment from pLG89 to a 0.9-kb Eco RI–Bam HI fragment from pLG83 and by inserting the 1.2-kb fragment into the Bam HI site of M13 mp8. This hph coding sequence fragment was then transferred into the Bgl II site of pMON237 (*8*) to create pMON404. This insertion created a chimeric CaMV 19*S* promoter–hph coding sequence–nopaline synthase 3′ polyadenylation signal (CaMV 19*S*-hph-NOS) gene in a pMON200 plasmid derivative. Plasmid pMON405 (not shown) carried the hph coding sequence in the reverse orientation and served as a negative control in the transformation experiments.

Leaf pieces cocultivated with *A. tumefaciens* carrying either the pMON404 or pMON405 vector began to form calli while on medium containing up to 30 μg of hygromycin per milliliter and all 12 calli assayed 9 days after infection with each of the vectors were found to be nopaline-positive, which indicated efficient transformation of cells in all of the leaf pieces. Two months later, after three transfers to fresh medium, calli were still growing on many leaf pieces transformed with pMON404 while only one callus from leaf pieces cocultivated with *A. tumefaciens* carrying pMON405 continued to grow on 10 μg of hygromycin per milliliter of medium (Table 1). The 405 callus did not produce nopaline, whereas all ten pMON404 calli tested did produce nopaline. Uninfected leaf pieces also occasionally produced calli in 10 μg of hygromycin per milliliter of medium, but never in the presence of higher concentrations. Similar results were obtained in replicate experiments. Thus, effective selection was obtained with concentrations of hygromycin of 20 μg/ml or higher.

Shoots emerged from the transformed callus within 3 months after inoculation. These shoots could be shown to be transformed by their resistance to hygromycin in the leaf callus assay, by their production of nopaline, and by DNA blot hybridization

Table 1. Transformation frequency of *Arabidopsis* leaf explants.

Expt*	Construct	Frequency in the presence of hygromycin at (μg/ml) 10	20	30
		Growing callus/total		
1	404	24/99		31/108
	405	1/30		0/30
2	404	42/52	30/53	24/56
	405	7/33	0/37	0/38
		Nopaline†/total		
3	404	5/5		5/5
	405	0/1		
4	404	4/4		4/4
	405	1/4†		

*Experiment 1: Leaf pieces were scored for growing callus 60 days after inoculation with pMON404 and pMON405 while on the stated levels of hygromycin. Experiment 2: Scored 34 days after inoculation. The number of leaf pieces with growing callus/total number of inoculated leaf pieces are shown. Experiments 3 and 4: A number of calli from experiments 1 and 2, respectively, were assayed for nopaline production (*5*) within 1 week of being scored for callus growth. Shown are the number of nopaline-positive calli per total number of calli assayed. †This positive was very faint.

Table 2. Progeny data. For the leaf callus assay, leaf pieces from sterilely grown seedlings were placed on callus-regeneration medium containing hygromycin (30 μg/ml) and scored for live calli 2 weeks later. For the seedling callus assay, see Fig. 3. With the exception of three opine-producing progeny from transformant A01, all opine-producing progeny assayed were hygromycin resistant and all nopaline-negative progeny were hygromycin sensitive. Plants maintained by self-fertilization labeled "self." Columns 2 and 3 show the number of nopaline-positive or hygromycin-resistant progeny per total number of progeny assayed. Wild-type, wt.

Plant source	Nopaline production	Hygromycin resistance
	Leaf callus assay	
wt	0/17	0/17
A01 self	33/33	30/33
wt × A01	5/5	5/5
wt × (wt × A01)	14/33	7/7*
		0/4†
(wt × A01) self	32/43	6/6*
		0/3†
A10 self	41/55	41/55
A15 self	12/17	12/17
	Seedling callus assay	
wt		0/44
(wt × A01) self		31/56‡
(A01 self) self		65/87‡

*Nopaline-positive progeny assayed for hygromycin resistance. Nopaline production was assayed as described previously (*5*). †Nopaline-negative progeny assayed for hygromycin resistance. ‡Thirty-five hygromycin-resistant seedlings from these two crosses were assayed for nopaline; all were positive. Hygromycin-sensitive seedlings could not be assayed for nopaline.

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analysis (Fig. 2). These shoots formed roots within 2 weeks on rooting medium (*12*) and were transferred to soil. For two out of three of these first transgenic plants, three-quarters of the progeny inherited the transferred DNA (T-DNA) as we expected from the Mendelian ratio for an inserted DNA carried in one chromosome of the parent plant. These progeny also demonstrated coinheritance of the selectable marker (hygromycin resistance) (Table 2 and Fig. 3) and the unselected marker (nopaline production) (Table 2). This coinheritance of an unselected marker provides formal proof that the hygromycin resistance was caused by transformation with the pMON404 vector rather than by an endogenous mutation. The first transgenic plant (A01) examined produced all nopaline-positive progeny (Table 2), a result indicating that it contained multiple, independent T-DNA insertions. In the next generation, plants containing single T-DNA's were readily identified.

Transgenic *A. thaliana* plants are readily obtained with the transformation-regeneration system described in this report. A key to the transformation system was the use of a selectable hygromycin-resistance marker. In our most successful experiments to date, approximately one-third of the original transformed leaf pieces gave rise to calli that survived the process of selection for hygromycin resistance; more than half of these regenerated shoots. The shortest time interval from inoculation of the leaf pieces to collection of seed from transformed regenerants was 4 months. Clearly, the efficiency of this transformation system will permit the introduction of natural and modified genes to study relative level and control of expression as well as the development of new markers.

The low incidence of repetitive DNA and small genome of *A. thaliana* suggest several potential uses for the more than 100 independently derived transgenic plants obtained with this procedure. These plants and their progeny could be used to test some of the proposed applications of randomly inserted T-DNA markers (*1*). A series of transgenic plants, each with the chromosomal position of the inserted T-DNA mapped by conventional genetics could be used for chromosome walking to closely linked genes with the T-DNA as a starting point. If the T-DNA's were within 200 kilobase pairs (kb) of a given gene (about five overlapping 40- to 45-kb cosmid clone fragments), then approximately 1600 independent inserts would be required to cover the entire 7×10^7 bp haploid genome. Since roughly 60% of the *A. thaliana* sequences are unique, it is likely that some of the T-DNA's have inserted into active

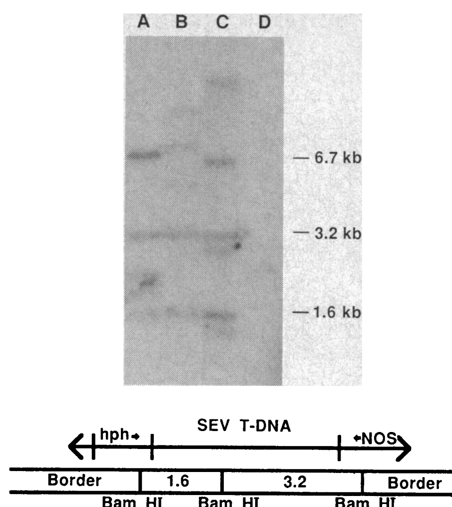

Fig. 2. DNA blot hybridization analysis of *Arabidopsis* plants. Total plant DNA was extracted by a modification of the method of Kislev and Rubenstein (*14*) with TESE buffer [50 m*M* tris, *p*H 8.0, 50 m*M* EDTA, 50 m*M* NaCl, ethidium bromide (400 μg/ml), 2% *N*-lauroyl sarcosine]. The DNA (10 μg) was digested with Bam HI, fragments were separated by overnight electrophoresis in a 0.7% agarose gel, transferred to nitrocellulose, and probed with nick-translated pMON404 DNA. (Lane A) Transformant A15; (lane B) transformant A10; (lane C) one progeny of transformant A01; and (lane D) wild-type DNA. The expected 3.2- and 1.6-kb internal Bam HI fragments appear in DNA from all three transformants. The multiple-border fragments indicate that there may be more than one T-DNA insertion in each of these plants. Below the blot is a schematic diagram of the expected T-DNA structure at the time it was inserted into the plant genome.

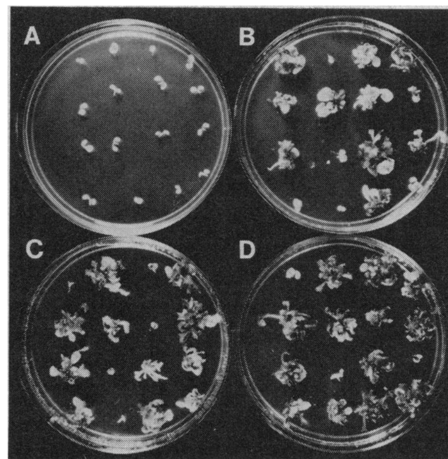

Fig. 3. Progeny of transformed and untransformed *Arabidopsis* plants germinated on callus-regeneration medium containing hygromycin (50 μg/ml). Each plate contains seedlings from a single silique (seed capsule). (A) The growth of wild-type untransformed seedlings is inhibited. (B) Progeny are seedlings of self-fertilized plant 5-1; 10 resistant, 6 inhibited. (C) Progeny are seedlings of self-fertilized plant 4-1; 11 resistant, 5 inhibited. (D) Progeny are seedlings of self-fertilized plant 3-7; 13 resistant, 3 inhibited.

genes. These gene insertion events may be identified by screening progeny of the transgenic plant lines. Multiple T-DNA inserts such as seen in one of our first transgenic plants will increase the chances of insertions into a gene of interest, but will require further genetic analysis to isolate the insert of interest. Demonstrations of the feasibility of these approaches to the identification, isolation, and analysis of specific plant genes will guarantee the position of *A. thaliana* as "the *Escherichia coli* of the plant kingdom" (*13*).

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