TRANSGENIC ARABIDOPSIS

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INTRODUCTION

A nuclear gene locus of (Arabidopsis thaliana L.) (Rédei, 1973; Rédei and Plurad, 1973) causes hereditary alterations in the genetic material of the plastids. Its effectiveness is quite remarkable in as much as the rate mutation when either one of the three known recessive alleles become homozygous, increases by a

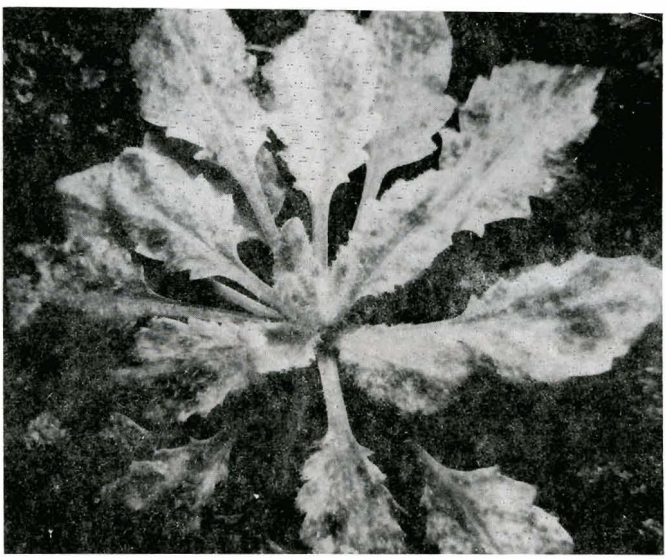


Fig 1. Plant homozygous for the chm^2 allele displays sectoring.

factor of about 10^6 over the spontaneous level. The mutator activity is revealed by the numerous green, yellow and white sectors on the leaves and stems of the plants (Figs. 1 and 2). Some of the mutations induced have pleiotropic effect: in addition to alteration of the plastids the shape of the leaves is also affected. Since the sorting out of the mutant plastids is clearly a non-random process, leaves or entire plants may become homo- or near homoplastidic within a single generation (Rédei, 1974). The homoplastidic condition generally cannot be stabilized, however, unless the recessive inducer is blocked by rendering the plants heterozygous or by the removal of the chm alleles from the nucleus. Details of these procedures were worked out and their effectiveness has been proven (Rédei, unpublished).

The mutator alleles are only slightly different in effectiveness, and all can induce both forward and apparent reverse mutation. In some of the mutant tissues the frequency of reversion is high (Fig. 3), in others it does not take place at

Fig. 2. Plants homozygous for the chm[1] allele illustrating some of the phenotypic effects of the mutator. Plant on the left shows uniform deformity of the leaves and sectoring, the individual in the middle has a large albina sector which -- albeit it grows as well as the parts with green pigments--does not display reversions, plant on the right indicates high rate of mutation and sorting out. Aseptic cultures like these permit better visualization of the pigment-free or low-pigment leaves.

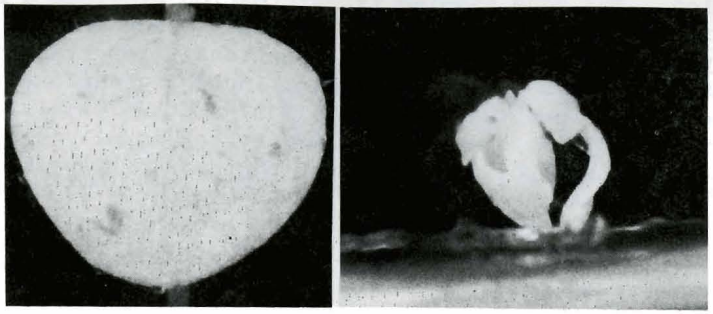


Fig. 3. Green sectors in an albina leaf (left) and in a small semi-lethal seedling (right) grown in vitro, indicating phenotypic reversions.

all (Fig. 2). The ability of back mutation does not seem to be related to the viability of the original genetic alteration. It appears that the mutator is capable of inducing both point mutations and losses, and it may be responsible also for rearrangements in the plastome.

At the time when the first such mutator was discovered (Li and Rédei, unpublished data), only classical genetic analysis and electron microscopic studies were feasible on the nuclear gene and its effects on the ultrastructure of the photosynthetic apparatus (Rédei, 1973; Rédei and Plurad, 1973). Recent advances in molecular genetics open new approaches to the analysis of the plastid DNA, and to the tagging and isolation of the nuclear gene itself. This study will be concerned with the development of techniques which eventually may lead to a physical characterization of the chm nuclear gene and its various mutant alleles.

TRANSFORMATION OF ARABIDOPSIS

The early studies of Barbara McClintock at the University of Missouri on chromosome breakage (McClintock, 1931; 1938) lead her to the discovery of the Ac - Ds system of maize (Zea mays L.) (McClintock, 1951) and to the genetic exploration of the controlling elements (McClintock, 1956). These mobile genetic elements could be identified in a variety of prokaryotic and eukaryotic organisms (Shapiro, 1983) and permitted the tagging of a number of genes with appropriate probes.

Arabidopsis does not have verified transposable elements albeit some mutations such as im (Rédei, 1963; Rédei et al., 1974) and chm bear some resemblance to them regarding the pattern of

variegation. For gene tagging transformation provides a better alternative because once an insertion takes place that is expected to stay put without additional manipulations. For transformation different procedures may be available, including various agrobacterial vectors (Márton et al., 1979; Otten et al., 1981; De Greeve et al., 1982), electroporation (Potter et al., 1984), direct gene transfer by plasmids into protoplasts (Paszkowski et al., 1984), injection of DNA into the shoot apices (de la Peña et al., 1987), and DNA-coated tungsten microprojectiles (Klein et al., 1987). Some of these procedures and applications have been briefly summarized recently (Cocking and Davey, 1987).

For our studies the techniques developed by Horsch et al. (1985) and applied to Arabidopsis by Lloyd et al. (1986) were adapted. This procedure, with modifications, have been successfully used by other laboratories (see 3rd Int. Arabidopsis Meeting, 1987) although Feldmann and Marks (1987) developed an entirely different method involving infection of germinating seeds by Agrobacterium carrying kanamycin resistance genes.

Vectors

The experiments discussed below used 6 different vectors developed by Csaba Koncz. Five of the vectors were free of the oncogenes of the Ti plasmids. The oncogenic vector was used only for testing the effectiveness of the system. For their overall features see Fig. 4. Vector pPCV 311, containing the oncogenic region, was the largest, approximately 22.4 Kb. It contained the neomycinphosphotransferase II gene (Km^R) attached to the nopaline synthase promoter (pNOS) and termination signals represented by the open boxes. It carried also the octopine synthase gene. The arrows indicate the directon of the transcription. B black boxes stand for the left and right border sequences of the T-DNA. pPCV 310 (approx. 14.2 Kb) is identical to pPCV 311, except it is free of genes, 5, 2, 1 and 4 of the tumor inciting plasmid. pPCV 631 has a hygromycin phosphotransferase gene of bacterial origin (Hyg^R) hooked to a nopaline synthase (pNOS) promoter and terminations signals and thus conveying resistance to the antibiotic hygromycin B. This vector does not contain the octopine synthase gene but it has the NPT II gene albeit without a promoter. Thus successful transformants become usually resistant to hygromycin but they can express kanamycin resistance only if the insertion takes place in such a way that the prokaryotic NPT II gene comes under the control of a plant promoter.

pPCV 601 is a vector with a promoter-free Km^R gene. Since under our experimental conditions transformants are screened on the basis of antibiotic resistance, isolation of transformants is very difficult because only those rare cases will be found which are integrated behind a plant promoter. Nevertheless a few

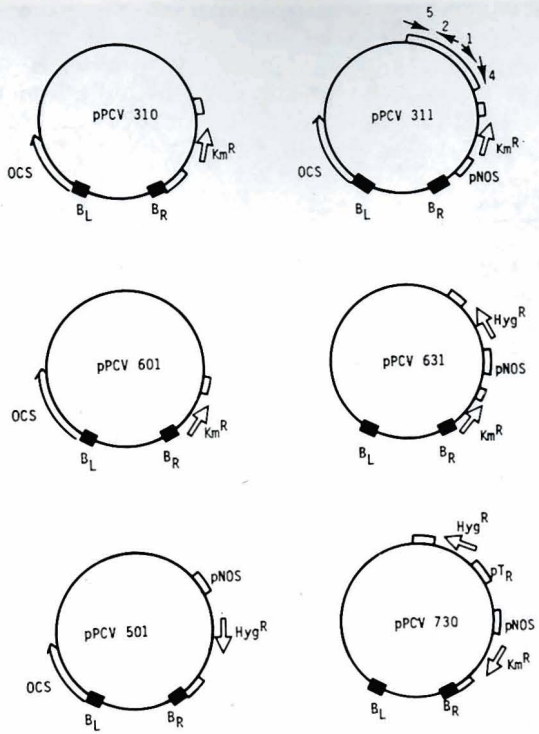


Fig. 4. Schematic representation of the 6 vectors involved. The diagrams are not on scale.

transformed cell colonies were obtained with this vector too. Transformants can be further identified by octopine synthase activity.

pPCV 730 carries both hygromycin and kanamycin resistance genes, equipped by a T_R and NOS promoter, respectively. Both genes can be freely expressed after integration irrespective of the site of insertion. This vector does not have an octopine synthase gene. pPCV 501 contains the hygromycin phosphotransferase gene in an opposite orientation; it contains the NOS promoter, and it has a functional octopine synthase gene. All these are binary vectors, and the VIR functions are provided in trans orientation.

In all experiments transformants were selected by either kanamycin or hygromycin resistance. Both of these antibiotics affect primarily the 16S ribosomal RNA (Moazed and Noller, 1987) yet their effect on Arabidopsis is quite distinct. Hygromycin seems to be a better selective agent than kanamycin although transformants have been obtained with both in our laboratories.

Hygromycin can entirely prevent growth of the plants without interfering with the synthesis or maintenance of leaf pigments. Kanamycin, at appropriate concentration, prevents the formation of all pigments and causes the bleaching of the tissues exposed. Their effects are also complicated by cefotaxime (claforan) when it is present in the culture medium.

Bacterial Cultures

Agrobacterium was grown on YEB medium (Lichtenstein and Draper, 1985) at 20°C with vigorous agitation. In about a day, high cell density was obtained. The cells were collected by centrifugation (Sorvall GS-3 rotor, 5,000 rpm, 10 min., 150 ml Corex bottle) and washed with tissue culture medium containing sucrose but no hormone or agar. For infection, the cell density was adjusted to about O.D. 0.8 at 550 nm.

The bacteria were stored on YEB masterplates containing 100 µg/mL rifampicin and carbenicillin, each, and they were kept in the refrigerator for about two months before reculturing.

Preparation of the Plant Material and Infection

Arabidopsis, Columbia wild type, was grown aseptically on E medium (Rédei, 1965) in cotton-plugged 16 x 150 mm test tubes under 12 hrs light cycles in a growth chamber at approximately 24°C or in the greenhouse. Illumination was provided by daylight fluorescent tubes at about 500 foot candle. The plants were used at the stage when the flowering stem reached to near half height of the inner space between the 5 mL medium and the enclosure Fig. 5.

From both rosette and stem leaves the base was cut off, and the stems were sliced into 10 to 20 mm long segments. The leaves were wounded with several cuts using sharp scalpels and the stem pieces were also pricked. The explants were then bathed for about 20 minutes in the bacterial suspension. After blotting the pieces, they were placed on tissue culture medium without hormones and incubated for two days at approximately 24°C.

Plant Culture Medium

As mineral salt solution, the nutrients suggested by Murashige and Skoog (1962) were used in some of the experiments. Much better regeneration was obtained by a new medium developed during the course of these studies, and this is now being used routinely for all phases of the transformation experiments.

The new nutrient solution (designated R4) contained the following ingredients in mg/L: NH_4NO_3 1,800, KNO_3 800, $MgSO_4 \cdot 7H_2O$ 100, $CaH_4(PO_4)_2 \cdot H_2O$ 100, KH_2PO_4 100, K_2HPO_4 90, Fe-pentetic 67.1. This latter component was prepared as a 0.02 M solution of $FeSO_4 \cdot 7H_2O$ and the chelating agent diethylenetriamine pentaacetic acid, and 5 mL was added to each L of the final medium.

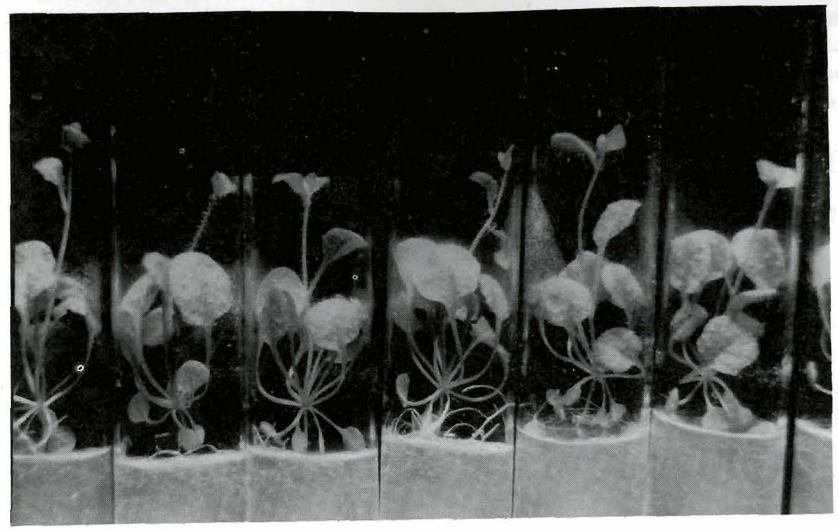


Fig. 5. Test tube-grown plants ready for bacterial infection.

The minor salt mixture was identical to that in the Murashige and Skoog solution. Vitamins were supplied as suggested by

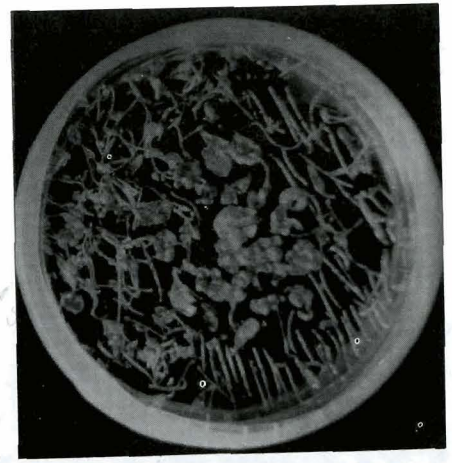


Fig. 6. The infected explants were laid on the surface of 30 mL nutrient medium in 100 x 10 mm sterile polystyrene plates and sealed with parafilm.

by Gamborg et al. (1968), except that in place of nicotinic acid nicotinic acid amide was employed. The medium contained also 3% sucrose, and it was solidified by 0.18% Gellan Gum (Gel-Gro, ICN Biochemicals, Cleveland, OH).

Proliferation of Transformed Cells

Two days after infection the explants were removed from the first solid medium where considerable growth of the bacteria has taken place and time was thus allowed for the integration of the T-DNA. The second solid medium contained cefotaxime (500 mg/L) to eliminate further bacterial growth which would seriously damage the plant tissue. Cefotaxime sodium is a semisynthetic cephalosporin antibiotic stops bacterial growth immediately but does not eliminate Agrobacterium cells until several passages have taken place. It is noticeably decomposed by two weeks. This antibacterial agent is relatively harmless to Arabidopsis cells, however, it interacts undesirably with aminoglycoside antibiotics (e.g. kanamycin). We have used a water-soluble powder obtained either as a gift from the Hoechst Company or purchased their product from Calbiochem, La Jolla, CA.

This second medium contained 2,4-dichlorophenoxyacetic acid (0.025 mg/L) and isopentenyl adenosine [6(γ,γ-dimethylallylamino) purine riboside, abbr. 9RiP] generally 1.5 mg/L. Depending on the selectability of the transformed cells, we generally used either 100 mg/L kanamycin sulfate or 15 mg/L hygromycin B. Calli developed to excisable size within about three weeks (Fig. 7).

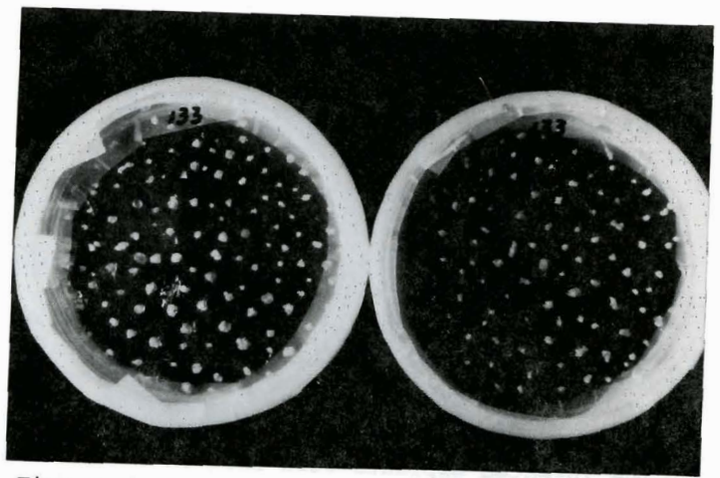


Fig. 7. Five-week old calli isolated on 100 mg/L kanamycin sulfate (pPCV 310).

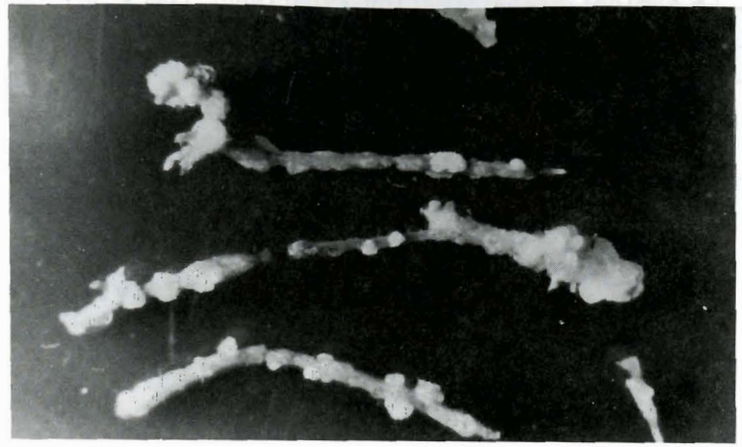


Fig. 8. Development of transformed cell colonies on infected stem segments (pPCV 310) placed on a medium containing 100 mg/L kanamycin sulfate.

The efficiency of the transformation was quite variable. In the experiment shown above from 583 explants 528 calli were obtained (ca. 0.91). In other experiments only a few percent successful transformation was observed. Generally, stem segments displayed considerably more antibiotic resistant calli than leaves (Fig. 8). The stem leaves were much more susceptible to transformation than rosette leaves. The age and vigor of the tissues were decisive factors. Some leaves developed several calli whereas others died shortly after infection.

The calli excised were subcultured every two to three weeks on shoot-inducing medium containing 2 mg/L 9RiP and 0.1 mg/L α-naphthalene acetic acid (NAA). Some of the calli developed leaves within a few weeks, for others several months were required to show leaf initials. After leaf initials appeared the dose of 9RiP was reduced to 1.5 mg/L. Within 5 months approximately 1/3 of the calli displayed leaves but the laggards still continued with leaf differentiation indefinitely. Nearly all the calli represented in Table 1 formed leaves and flowering stems after 8 months.

After a few weeks the level of cefotaxim in the culture medium was reduced to 200 mg/L and maintained this way until the bacteria were eliminated. After bacterial infection of the cultures ceased cefotaxim was omitted from the media. Following a few subcultures kanamycin and hygromycin, respectively were

Table 1. A typical transformation experiment (pPCV 501) yielded the results shown below.

		%
Total of leaves and stems infected	1977	100
Surviving calli by 5 months	701	35.5
Calli differentiating only leaves by 5 months	128	6.5
Plants with flowering stem obtained	190	9.6

discontinued in the media to assure more vigorous growth. Although the transformed calli tolerated these antibiotics, they did better without them.

Differentiation of Complete Plants from Transformed Calli

The plantlets seen on Fig. 9 eventually form a flowering shoot on 1.5 mg/L 9RiP and 0.1 mg/mL NAA. In some experiments better shoot formation resulted on 0.5 mg/mL 6-benzylaminopurine (BAP) and 0.05 mg/mL NAA. At this stage some of the plantlets we transferred into test tubes of various sizes to allow better

Fig. 9. When leaf differentiation begins, the majority of the calli form multiple plantlets. Because of the high level of cytokinin, the leaves are generally purplish or deep purple but soon after this hormone is reduced they turn green again and the leaf blades assume wider normal shape.

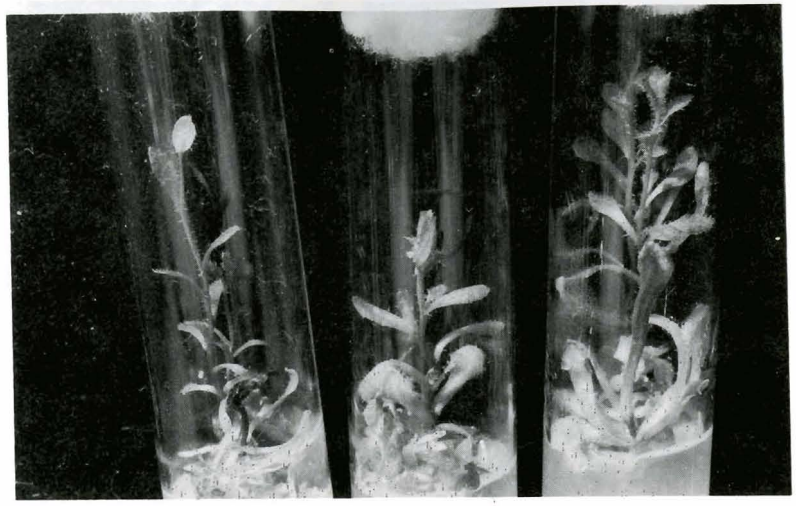


Fig. 10. Transgenic plants at the shooting stage in test tubes.

growth (Fig. 10). Particularly favorable were the 20 x 150 mm test tubes. Some of the plantlets formed viable seeds within the test tubes, obviating the need for further transplantation.

Unfortunately, very few of the plants developed roots under these culture conditions. On aseptic media, the presence of roots was not absolutely necessary for seed production. Rooting was attempted by treatments with various auxin and cytokinin combinations and single or mixed treatments of indoleacetic acid and indolebutyric acid but no really satisfactory solution is available to this problem.

Many initially rootless plants thrived well when transplanted from the axenic culture to an artificial potting mix (Pro-Mix BX), and produced seeds. Some of them fed only through the calli still attached to the stem, others rooted spontaneously. Attempts to root the plants by dipping the base into "Rootone F" (Union Carbide) commercial rooting agent controlled to some extent fungus infection but showed no other beneficial effect. Actually, many transplants bleached as a consequence of this treatment and perished.

Some transformants produced well over 1000 seeds while others yielded only a couple. The percentage of viable seed per silique varied a great deal but the majority of seed was of good quality and germinated well. The appearance of the regenerated transformants was quite different from seed-grown ones or from the much faster regenerated individuals which were not involved

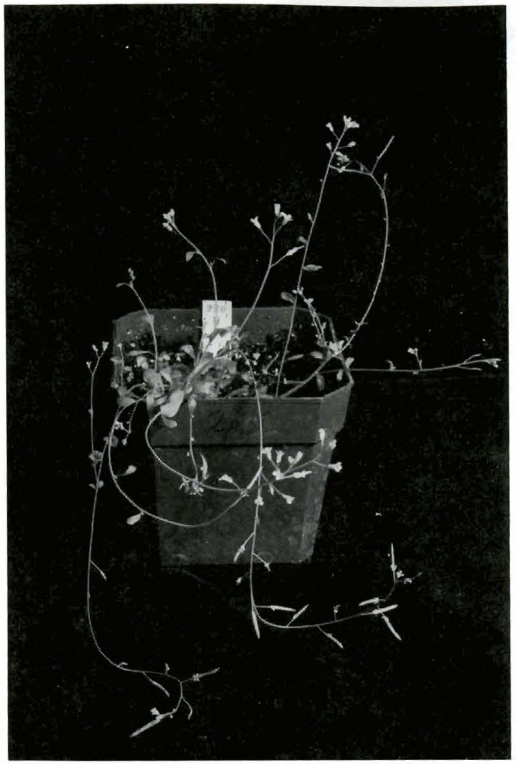


Fig. 11. Regenerated plant of typical appearance displaying fruits and flowers. Frequently, these type of plants continued developing new shoots after this stage and produced many more fertile fruits.

with bacterial infection and the accompanied treatments with antibiotics. Most of the transformed plants displayed a bushy habit (Fig. 11) with a larger number of stems than the regularly regenerated ones and certainly many more than those originating from seed.

VERIFICATION OF TRANSFORMATION

Although all the calli came through several passages of selective media, some regenerated individuals turned out to be susceptible to the antibiotics when the progeny was germinated on hygromycin or kanamycin media, respectively. So far only a small number of plants were tested, therefore the percentage of escapees cannot be accurately or meaningfully determined. Generally, the seedlings are more sensitive to the selective antibiotics than the coresponding calli.

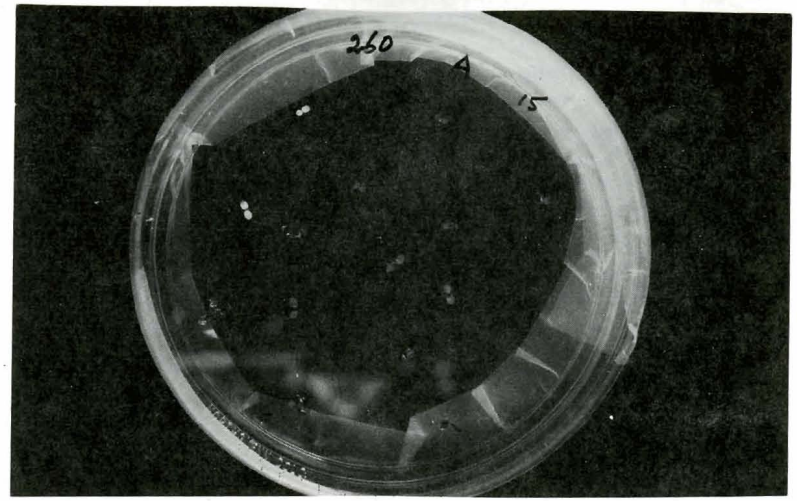


Fig. 12. T_2 progeny of plant 310-A tested on 60 µg/mL kanamycin sulfate; 14/15 seeds planted germinated and 12 (dark green cotyledons) appeared resistant and 2 (pale) was found to be susceptible to the antibiotic.

Progenies of Transgenic Plants

So far seed was obtained on transformants infected by pPCV 310, pPCV 501 and pPCV 730. Only small progenies were tested for segregation (Fig. 12 and 13). The proportion of the resistant seedlings indicated that the most common occurrence was where more than one copy of the T-DNA was inserted into the chromosomes of the plants. In all T_2 populations where less than 20 individuals were tested there was at least one susceptible plant, suggesting that no more than two nonhomologous chromosomes were involved in the transformation of the original cells. The segregation ratios did not provide precise information on the number of inserts because it was impossible to tell why in some progenies a large proportion of the seeds failed to germinate. The non-germinating seeds might have been the result of physiological conditions prevailing during the differentiation and development of the reproductive structures. It was also conceivable that the dead seed contained insertions into vital genes and thus suffering from dominant lethal mutations.

Only 13 seeds were planted of plant 501-13 (pPCV 501) from which 2 failed to germinate, 2 were obviously resistant to hygromycin B, 2 were pale, and 7 albina showed up. This indicated that insertional mutations may have occurred. These mutations may have had different phenotypic consequences in the heterozygous and homozygous condition and they may have epistatic interactions.

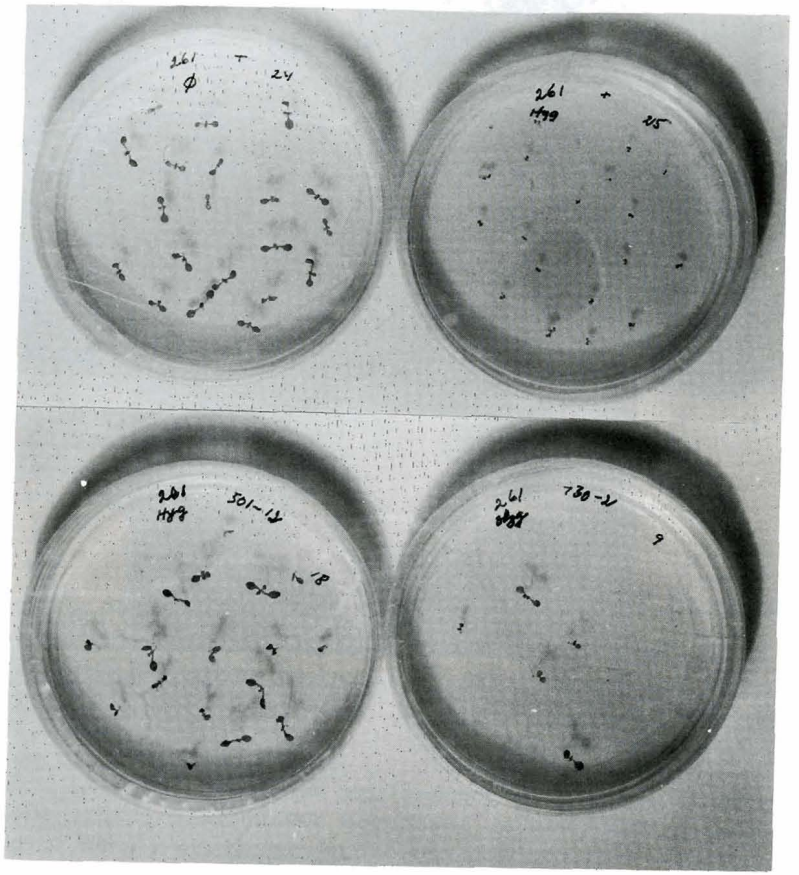
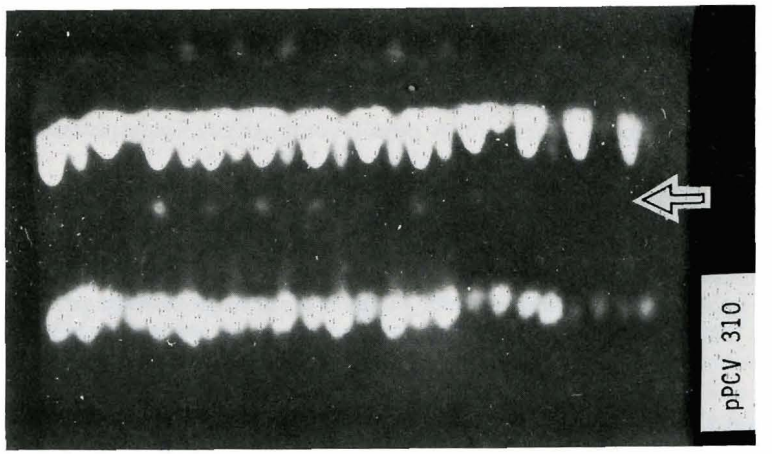


Fig. 13. Hygromycin B even in minute quantity (2.5 μg/mL) has clear effect on Arabidopsis at the cotyledonous stage. Top: wild type plants germinated without hygromycin (left), and on hygromycin containing medium (right). Bottom: T_2 progenies of transgenic plants showing segregation for hygromycin resistance. Left: transformed by pPCV 501, right: by pPCV 730. Note the difference in the size of the cotyledons. Even more conspicuous was the difference in the elongation of the roots (not visible on the picture because of the white background).

Biochemical Evidence of Transformation

Octopine tests. Of the six vectors used, four contained information for octopine synthesis. Octopine is an arginine analog which can be produced only by plant cells which were transformed by appropriate Agrobacterium strains carrying the specific T-DNA gene (OCS). The analysis was carried out by the procedure of Otten and Schilperoort (1978) as modified by Murphy and Otten (1985). Small callus pieces are sufficient for the detection of this opine. From each plant assays were made both with and without the complete reaction mixture. The material examined revealed octopine from the complete system only. After four hours of incubation aliquots were separated by paper electrophoresis and the resulting fluorescent spots on phenanthrenequinone-treated paper were photographed under ultraviolet light by a polaroid camera (Fig. 14). Some of the octopine spots were quite faint yet other line of evidence (neomycinphosphotransferase II activity, Southern blots) indicated that the same plant was transformed indeed.

Neomycinphosphotransferase assays. The aminoglycoside, kanamycin may have several different effects on the ribosomes (Moazed and Noller, 1987), among them the most important is probably the inhibition of amino acid translocation by attaching



1 2 3 4 5 6 7 8 9 10 11 12

Fig. 14. The majority of the transformed tissues provided extracts with detectable octopine synthase activity. Octopine appeared as small fluorescent spots (arrow). The large spot above octopine is arginine, and below NADH, both ingredients of the reaction mixture.

to the A site and also by interfering with decoding by sequestering critical macromolecules and distorting conformation of structural domains necessary for protein synthesis. Umezawa and coworkers (1967) showed 20 years ago that a gene carried on an R plasmid of Escherichia coli was capable of inactivating several aminoglycosides by virtue of phosphorylation at critical and specific hydroxyl groups. A single phosphorylation may suffice to eliminate most of the toxicity of this group of antibiotics. The phosphokinase with a narrower spectrum of specificity was called neomycinphosphotransferase I, and the other capable of adding phosphates to an additional class of molecules was named neomycinphosphotransferase II (see Haas and Dowding, 1975).

The vectors used in these studies conveyed kanamycin resistance to the transgenic plants because they contained the structural gene of NPT II derived from Tn5 of E. coli. NPT II activity was assayed according to the procedure developed by Reiss et al. (1984) with modifications by Schreier et al. (1985). Plant extracts were subjected to discontinuous polyacrylamide gel electrophoresis. Subsequently, the gel was overlayered--on a sealed glass plate--with a 1% agarose containing 1 mg/mL kanamycinsulfate and 100 µCi ATP in an incubation buffer. The solidified agarose was then overlayed by 1 sheet of P81 phosphocellulose paper and 3 sheets of 3MM filter paper and on top of them a pile of blotting material was weighted down. Then a 3 hr incubation took place. After this the P81 paper was deproteinized enzymatically in a sealed bag at 60°C (1 mg/mL protease in 1% SDS). The phosphocellulose paper was then washed several times by warm 10mM phosphate buffer, pH 7.5. After drying it was autoradiographed. The bottom spots represent the phosphorylated kanamycin molecules, and thus neomycinphosphotransferase activity. The spots above show some other unspecific phosphokinase activities of the plant extracts (Fig. 15).

Presence of Foreign DNA in the Genetic Material of the Plants. From Ti plasmid vectors only the T-DNA, bounded by 25-base pair direct repeats, is integrated into the plant chromosome(s) (Wang et al., 1987). The presence of intact left and right border sequences (Fig. 4) were essential for the facile insertion of the passenger DNA. The integrated T-DNA retains most of the bases of the left border sequences frequently whereas generally there is no junction beyond the third base of the right border (Wang et al., 1987).

The integration is mediated by the virulence loci (vir) located on the bacterial plasmid and chromosome (chv). The genes were activated in response to plant wound signal molecules of acetosyringone.

For the detection of the physical presence of the foreign

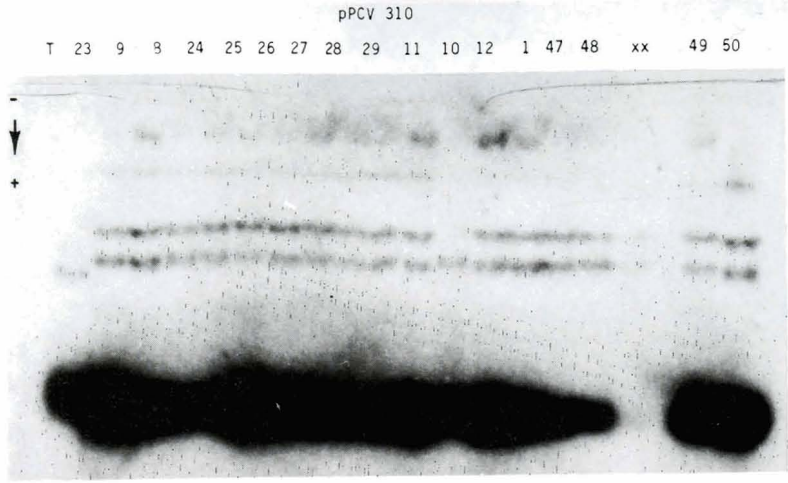


Fig. 15. Autoradiogram of the products of a neomycinphosphotransferase assay. The first lane (T) at the left shows NPT II activity from transgenic tobacco tissue. All other lanes are from Arabidopsis plants transformed by pPCV 310 (except the xx control lane). Altogether 37 plants were assayed and all appeared the same. Note the difference in mobility of the unspecific kinases in tobacco and Arabidopsis (in the middle of the photo).

DNA, Southern analysis was used (Southern, 1975). As probes both the left and right ends of the T-DNA were employed, including the octopine synthase (OCS) and the neomycinphosphotransferase (NPT II) genes, respectively (Fig. 16).

All five plants--unexpectedly--have the same fragment pattern. This may be a fortuitious coincidence or it may be that they were derived from the same original transformed cell. The fast growing calli can be quite friable at the early stages and they all may represent the same tissue clone. These plants originated from the very first attempts at transformation of Arabidopsis in the laboratory.

All five plants appeared to have two inserts. This information was supported by the genetic observations, that none of the T_2 progenies tested segregated for 3 resistant: 1 susceptible to kanamycin or hygromycin, respectively. Rather, approximately digenic ratios were observed (Fig. 13). Several more plants must be analyzed and additional restriction enzymes must be used before further conclusions are made. It is obvious that the OCS and the NPT II genes were actually incorporated into the plant chromosomes.

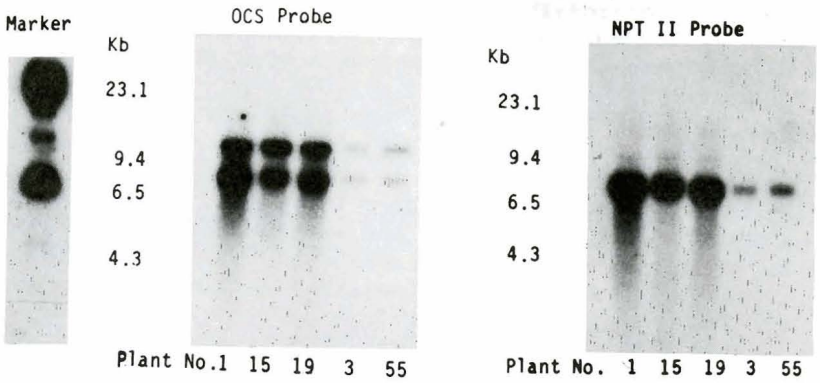


Fig. 16. Southern blots of transgenic Arabidopsis plants (pPVC 310). The molecular weight marker is Hind III digested phage lambda DNA. Arabidopsis DNA was digested also by the same restriction endonuclease.

GENETIC ANALYSIS OF THE TRANSGENES

At the moment the locations of the transgenes in the Arabidopsis genome are not known. The chromosomal assignment of the inserts can be made most economically by trisomic analysis. Fortunately primary trisomics are available for all five chromosomes of the plant genome, in addition one telotrisomic can be used (Lee-Chen and Steinitz-Sears, 1967). The primary trisomics are phenotypically distinguishable from the disomic plants and from each other, and they can be identified without chromosome counts (Fig. 17).

Assignment of Inserts to Chromosomes

The transgenes do not have counterparts in the plant genome, and they are expressed as dominant mutations. In the case where more than one insert is within a particular chromosome, trisomic analysis can not determine their location unless they were situated on different arms and telotrisomics would be available for at least one of the two arms concerned. The advantage of using trisomic analysis over test crosses or the product method (Rédei, 1982) is the lack of ambiguity regarding the chromosome involved. Actually, making only four different crosses suffices; if an insert is not in one of the four, it must be in the fifth. In the case where conventional crosses are used, and linkage is sought with strategically positioned markers along the chromosome, the clarity of the results are influenced by the frequency of recombination between the markers involved and the insert(s). The analysis may be particularly cumbersome when plant color markers

Fig. 17. The primary trisomics of Arabidopsis on Columbia wild type background grown under natural summer day length in Missouri. At the upper left corner is a normal disomic plant. The appearance of the telotrisomics available is very similar to the plant shown in the upper right corner, and it is trisomic for only one arm of the same chromosome.

are used that may mimic the effects of the selective media on the susceptible segregants.

The simpliest procedure is to cross the trisomics by transgenic plants and study only the progeny of the trisomic individuals from the first generation. The transmission of the extra chromosome is generally limited to the egg, and even through the egg, transmission is less than the 50% theoretically expected. Thus, among the offspring of trisomics no more than 1/5 or 1/10 of the individuals are trisomic. Obviously trisomics can produce two main types of gametes: monosomics and disomics. The proportion of the chromosome markers is also affected by the distance of the markers (in this consideration the insert) from the syntenic centromeres. Longer distances permit more frequent recombination between the insert and the centromere and favors the higher frequency of identical segments in the same gamete (Table 2).

The theoretical expectations may not be realized among the gametes actually formed because the inserts may affect the transmission of the chromosome involved. Heterozygosity for the insert is not a problem in the initial cross but in such a case only half of the trisomic offspring will be useful for the

Table 2. Gametic output of trisomics (Iii) with one insert (I); i stands for lack of an insert.

Insert is absolutely linked to the centromere				
II	Ii	ii	I	i
0	2	1	1	2
Insert segregates independently from centromere				
II	Ii	ii	I	i
1	6	5	4	8

analysis. Since the male transmission of disomic gametes is always much reduced or nil, the trisomics must be used as the pistillate parent.

Since the genetic constitution of the gametes cannot be directly determined, self-pollinated progeny of the trisomics must be used for the analysis. Knowledge of the gametic expectations is necessary to predict the sporophytic proportions (Table 3).

The expected segregation ratios can be easily distinguished from the 3 I : 1 i, expected when the insertion does not involve

Table 3. Phenotypic proportions among the progeny of trisomics heterozygous for an insertion (Iii). The numbers refer to the phenotypes only and disomic and trisomic individuals are pooled on the basis of presence or absence of the insert. Albeit six different possibilities were considered, under practical experimental conditions intermediate situation between the last two rows of the table are expected to occur most commonly.

Transmission of disomic gametes through	Absolute linkage I	i		Independent segreg. I	i	
Male and female	3	1	(0.25)	2.41	1	(0.29)
Female only	2	1	(0.33)	1.77	1	(0.36)
Neither male nor female	5	4	(0.44)	1.25	1	(0.44)

Table 4. Gametic output of recessive insertional mutations (AAa).

The mutation is absolutely linked to a centromere				
AA	Aa	aa	A	a
1	2	0	2	1
The mutation segregates independently from centromere				
AA	Aa	aa	A	a
5	6	1	8	4

the chromosome present in three doses. Even the worst scenario of 2 : 1 segregation of the trisomic's offspring is distinguishable from the 3 : 1 at the 0.95 probability level in a population of 133. Once the synteny is determined, the more precise chromosomal position can be sought with the aid of the available chromosomal markers. Wallroth et al. (1986) used for transformation Petunia hybrida with at least one genetic marker on each of the seven chromosomes.

Chromosomal Assignment of Insertional Mutations

Insertion of the T-DNA within the boundaries of plant genes may result in inactivation of that gene detectable as recessive mutations. These recessive mutations can also be assigned to chromosomes by trisomic analysis even easier than the inserts as long as the insertional mutations have clear stable phenotype.

The combined disomic and trisomic offspring of heterozygous trisomics reveals the chromosomal location of the insertional recessive mutations. When the recessive mutation does not involve the chromosome triplicate in the trisomic, the phenotypic proportions are 3 normal wild type : 1 recessive mutant. When the trisome carries the mutation the segregation ratio varies from between 17 : 1 to 8 : 1, depending on its vicinity to the centromere, and even more importantly on the degree of transmission (Table 5).

When synteny is known, the situation of the new mutation within the linkage group can be determined by standard procedures. In cases where there are no markers within less than 20% recombination of the mutations vicinity, Haldane's mapping function may be useful for more precise mapping (Rédei, 1982). Mapping inserts and insertional mutations is of considerable interest because it may reveal much about the organization of chromosomes. Arabidopsis has only 1 - 3% as much DNA as the common angiosperms, and it is particulary low in redundant sequences (Pruitt and Meyerowitz, 1986).

Table 5. Phenotypic proportions in the progeny of trisomics of AAa genetic constitution.

Transmission of disomic gametes through	Absolute linkage A	a		Independent segreg. A	a	
Male and female	35	1	(0.03)	22.04	1	(0.04)
Female only	17	1	(0.06)	13.40	1	(0.07)
Neither male nor female	8	1	(0.11)	8	1	(0.11)

DISCUSSION

Transformation of Arabidopsis by the use of various agrobacterial vectors does not pose any more problems. At this time over 16,000 calli selected on hygromycin and kanamycin media, respectively are available. More than 12,000 differentiated rosette leaves or they are at the flowering or seed-bearing stages.

Hygromycin was found to be an effective selective agent (Lloyd et al., 1986), but it appears now that transformation to kanamycin resistance is also practical with the leaf or stem infection methods. During the course of this study much simpler procedures than previously used (such as feeder cells) proved to be quite effective. The mineral media and hormone regimes reported in this paper eventually result in almost 100% shoot regeneration on the surviving calli. Shoot formation occasionally is detectable on the 9th day of the cultures.

One serious bottleneck in the process still remains, namely the lack of a reliable procedure for rooting the shoots. Frequently, high frequency root differentiation adversely affects the formation of normal fruits and viable seed. However, successfully rooted plants produce more and better quality seed. If this last hurdle can be overcome, the procedure of transformation of Arabidopsis may be suitable to targeting specific genes with inserts.

According to an estimate the number of gene loci of Arabidopsis capable of yielding visible mutations is approximately 28,000 (Rédei et al., 1984). In case insertional mutations occur at random, and uniformly along the entire genome of Arabidopsis,

each of these loci have about 1/28.000 (ca. 4×10^{-5}) chance for being hit by the T-DNA. Since the size of individual genes, just as mutability, varies over a wide range, the probability of specific insertions can be expected to differ as well. Ignoring the extremes, and assuming average chances one can estimate the probability of inflicting a particular gene with an insertion. In order to find at least 1 inactivation by insertion at a chosen level of probability one must solve the following equation:

$$f^n = p$$

or it can be rewritten in a more meaningful way because one wishes to determine the size of the population (n) required where among 28,000 potentially hit genes there would be no more than 5% chance of missing one desired type of alteration: $(27{,}999/28{,}000)^n = 0.05$ hence $n = \log 0.05/(\log 27{,}999) - (\log 28{,}000) = 83{,}883$. In other words, if the chances for all genes are the same for being hit by an insertion, among ca. 84,000 insertional mutation cases at least 1 must be the desired type with a probability of 0.95. Since the transformation experiments are carried out with diploid tissues, 42,000 cells may suffice.

In our transformation experiments the number of transgenic calli obtained approached this figure. In addition, there is an indication for the presence of insertional mutations among the transgenic plants in our as well as in the experiments carried out by Alan Lloyd (personal communication). Thus tagging even specific selected genes may be within reach in extended experiments.

There is also another possibility by gene tagging through the use selectable structural genes introduced into the plants without appropriate promoters. The fusion of the selectable (reporter) gene with other genes permits the identification of the other component as well as their genetic and developmental regulation (André et al., 1987). Plasmids pPCV 601 and 631 used in these studies can be employed for such analyses.

SUMMARY

Although Arabidopsis was recalcitrant to transformation for a number of years, the production of transgenic indivdiuals became quite practical within the past year. Transformation was accomplished with six different binary vectors conveying one or two of the following functions to the plant cells: octopine synthesis, neomycinphosphotransferase II and hygromycinphospho-transferase. Over 16,000 transgenic calli were selected and 12,000 of them could be regenerated into shooty plants. The success of transformation was verified by selective growth, octopine assays, neomycinphosphotransferase II activity, Southern

hybridization and analysis of the seedling progenies. The procedures used in infection, for tissue culture and regeneration are reported. The procedures for assigning inserts and insertional mutations to chromosomes are outlined and the problems involved in gene tagging by T-DNA are discussed.

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