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Facile transformation of *Arabidopsis*

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Summary. A protocol is described for the simple, rapid and efficient production of transgenic *Arabidopsis* plants. The procedure was developed using growth regulator regimes that promote adventitious embryogenesis during or immediately following *Agrobacterium* mediated transformation. Both the RLD and Columbia genotypes of *Arabidopsis* were transformed using slightly different growth regulator regimes. For the Columbia genotype two modifications of the protocol were identified which substantially improved regeneration. Cold treatment of the plants used as a source of root explants resulted in a three-fold increase in the number of morphogenic sectors produced. A more important modification was the inclusion of 25 mg/l silver nitrate (an inhibitor of ethylene action) to the medium used for shoot regeneration. This provided a ten-fold increase in the number of shoots produced. These procedures made it possible to obtain over 100 putative transformants of RLD or Columbia from a single 10 cm petri dish, within 2 or 4 weeks after exposure of root explants to the bacteria. When these were transferred to rooting media containing antibiotics, approximately 20% were able to root after kanamycin selection and 80% after hygromycin selection. All the rooted plantlets tested were shown to contain integrated donor DNA as determined by Southern blot analysis.

Introduction

Arabidopsis thaliana is a flowering plant that is now widely used in genetic and molecular studies. The advantages of *Arabidopsis* for classical genetics include its small size and short generation time. Because the plants produce their first seed in less than six weeks, generations can be rapidly advanced. The plants are self-fertile and outcross at a very low frequency (<0.1%). However, the flowers can be easily emasculated to permit crosses between different lines (Rédei 1975). Despite its small size, *Arabidopsis* is a typical dicotyledonous plant and has been the subject of many physiological and biochemical studies (Estelle and Somerville 1986, Meyerowitz 1989).

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The considerable genetic information about *Arabidopsis* that has been obtained over the last 50 years forms an excellent foundation for molecular biology. However, it is mainly other attributes of *Arabidopsis* which account for the large increase in the number of laboratories using this tiny weed for molecular approaches to problems in plant biology (Chang *et al.* 1988, Meyerowitz 1989). These include a small nuclear genome, which reduces the effort required to screen genomic DNA libraries, a very small amount of dispersed repetitive DNA and a high percentage of single copy sequences.

Nevertheless, there are several aspects of *Arabidopsis* biology that detract from its reputation as the ideal model in plant molecular genetics. Chief among these is the fact that *Arabidopsis* is difficult to transform. Although methodologies developed over the last five years allow the routine production of transformants, the efficiency is often low. This is especially true for the race Columbia, which has been used as a wild type in many genetic studies (Rédei 1975). An additional problem is the relatively long time required to recover transgenic plants. Techniques based on cocultivation of leaf disks, stem or hypocotyl segments or seeds require at least four months (Lloyd *et al.* 1986, Sheikholeslam and Weeks 1987, Rédei *et al.* 1988). Methods based on the use of root explants are now available and, for these, the regeneration time is typically eight to twelve weeks (Valvekens *et al.* 1988, Koncz *et al.* 1990).

In order to provide a more rapid and efficient procedure, we have reevaluated each step in the transformation-regeneration process. Here we describe a protocol which provides substantial advantages both in the number of regenerated transgenic plants produced and the time taken to produce them.

Materials and methods

Seeds of Arabidopsis thaliana (genotypes RLD and Columbia) were kindly provided by Dr.C.R. Somerville (Michigan State University). The Ach5 Agrobacterium tumefaciens strain (carrying the pAL4404/-pMOGEN24 binary construction) was used in all transformation ex-

periments. The PMOGEN24 construction which contains the P_{35S}-hpt-nos3' and the P_{nos}-npt-ocsnos3' cassettes between the T-DNA borders was provided by Dr. P. Van der Elzen, Mogen Co., Leiden. The PMOGEN24 plasmid was transferred into the LBA4404 host (Ach5 carrying the PAL4404 helper plasmid, Hoekema et al.1983).

Media. **G-H**: Gamborg's B5 salts +1% sucrose and 1% agar (Gamborg et al. 1968). **MS-H:** MS salts +3% sucrose (Murashige and Skoog 1962). **ARM I**: MS salts, 3% sucrose, 0.8% agar, 2 ml/l vitamix (100 mg myo-inositol, 5 mg vitamin B1, 0.5 mg vitamin B6, 0.5 mg nicotinic acid, 1 mg glycine, 0.05 mg biotin per ml). Hormones: 3 mg/l IAA (indoleacetic acid) 0.15 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid), 0.6 mg/l BA (benzyladenine) and 0.3 mg/l IPA (isopentenyladenine). **ARM IIr**: As ARM I but hormones: 0.2 mg/l NAA (naphthaleneacetic acid), 4 mg/l IPA. **ARM IIc**: As ARM I but hormones: 0.1 mg/l NAA, 2 mg/l IPA, 1 mg/l IPAR (isopentenyladenosine), and 25 mg/l silver nitrate if it was applied. **ARM III:** As ARM I but hormones: 0.5 mg/l IBA (indolebutyric acid), 0.2 mg/l zeatin, 1 mg/l IAA (if GA_3 [gibberellic acid] was added, 1 mg/l was used). All media were autoclaved 20 min at 120°C even if silver nitrate was used (in ARM IIc medium). The addition of silver nitrate resulted in a fine, colloidal precipitate with other anions present in the medium (chloride, phosphate, etc.). However cleared ring appeared around the inocula, demonstrating that silver was mobilized by the tissue. Only IAA, IPA, IPAR, IBA, GA_3 and the antibiotics were filter sterilized and added to cooled media. The Gamborg and MS basic salts were supplemented with 3 ml of Miller's solution (6% KH_2PO_4). All medium salt mixtures and other components including kanamycin and hygromycin were obtained from Sigma. The Claforan (cefotaxime) was obtained from Hoechst, the Geopen (carbenicillin) from Roering.

Growth of plants. Prior to germination on G-H medium, seeds were sterilized (2 min in 70% ethanol, followed by 3 min in 10x diluted commercial bleach [0.5% hypochlorite]) and washed 4 times in sterile water. The sterilized seeds were spread on the surface of the G-H medium in 3 ml water. After the seeds were evenly distributed, the water was removed and the petri dishes were maintained at 24°C under a 16 h photoperiod (800-900 lux) in a growth chamber. Three weeks later, the plants were transferred to the coldroom for cold treatment and storage for at least two weeks (4-6°C, continuous dim light, about 100 lux).

Cocultivation and regeneration. Plants were transferred to the culture room (27°C, 16 h photoperiod, 900-1000 lux) for 24 h prior to the preparation of root explants. The agar disk was then turned over to expose the roots which were collected and transferred onto ARM I medium. For cocultivation, the roots, which had been induced on ARM I (3-5 days for RLD and 5-10 days for Columbia type), were wounded by cutting into 0.5-1 cm sections or by squeezing firmly with forceps at 2-5 mm intervals. About 100-200 cm of induced and wounded root explants were cocultured for two days in 10 ml of MS-H containing 0.25 ml of an overnight culture of Agrobacterium (Márton et al. 1979, Márton 1984). In certain cases, 20 μM acetosyringone was used to induce the bacteria (Sheikholeslam and Weeks 1987).

At the end of the cocultivation period, the bacterial mass was removed by rinsing in fresh MS-H and by pulling the roots through solid G-H medium. The explants were transferred onto ARM I medium containing 200 mg/l each of claforan and carbenicillin. After two days, the explants were transferred onto ARM IIc (for Columbia) or ARM IIr (for RLD) medium containing 50 or 100 mg/l kanamycin or 10 or 20 mg/l hygromycin. After 2-4 weeks, the putative transformants were scored for resistance, and suitable plants were transferred onto ARM III medium containing 100 mg/l kanamycin or 20 mg/l of hygromycin to screen for root induction. (Carbenicillin and claforan were present in all culture media used after the cocultivation step).

DNA isolation and Southern analysis. Genomic DNA was isolated by a mini version of the cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich 1985).

The genomic DNA samples (3-5 μg) were digested by restriction endonucleases, separated by standard agarose gel electrophoresis, blotted onto Zeta-probe membrane (Bio-Rad) and hybridized as recommended by the supplier. The probe was a P_{35S}-npt-nos3 fragment liberated from the pCaMVNEO plasmid by HindIII digestion (Fromm et al. 1986) and labeled by random priming. Membranes were washed according to the manufacturer s recommendations except that prior to the high stringency washing step, 2% fish gelatin was added to the standard washing solution. This resulted in a considerable reduction in background radioactivity.

Results and Discussion

Previous investigations have shown that root explants of *Arabidopsis* are excellent starting material to produce transgenic plants *in vitro* by *Agrobacterium* cocultivation (Valvekens *et al.* 1988). Preliminary results confirmed that root material was optimal in our protocol as well. However, when we used the protocol described by Valvekens *et al.* (1988), it took at least three months to regenerate plants and the efficiency was low - approximately one plant per 2-3 cm of original root explant in the case of the RLD genotype. In the Columbia genotype, 3-4 times fewer plants were obtained. When we used alternative hormone regimes (Rédei *et al.* 1988, Koncz *et al.* 1990), some improvement in regeneration efficiency was achieved. We, therefore, reevaluated each step of the regeneration/transformation process in order to provide a more rapid and efficient procedure.

Low temperature pretreatment

Cold treatment has occasionally been used to improve regeneration of plants from *Arabidopsis* tissue cultures (Negrutiu and Jacobs 1978). We attempted to use this approach to improve the morphogenic response in root explant derived cultures. However, exposure of cultures to 5°C for two days during either the culture induction phase or the regeneration induction phase, did not result in any significant change in the number of plants recovered. Instead, we found that prolonged cold storage of the seedlings at 5°C prior to harvest of the roots, lead to a pronounced increase in the number of the morphogenic sectors produced. The morphogenic sectors can be identified as compact polymeristematic sectors with embryoid structures (Fig. 1B, Fig. 2A,B,C). From each morphogenic sector 5 to 10 shoots could be harvested, especially when the larger shoots had been removed (a kind of "apical dominance" could be seen). Since those were considered to be amplification products of a single initial transformation and/or embryogenesis event, we counted only one plantlet from each sector.

Even without cold, treatment 4-5 week-old plants of the RLD genotype provided roots that gave rise to more than 30 morphogenic sectors per cm. Although cold treatment was not required to improve the regeneration of the RLD genotype, the cold storage of seedlings in the original cultures provided a convenient means of ensuring a continuous supply of root material. In contrast to the results obtained with RLD, roots from the Columbia genotype responded poorly to the morphogenic conditions when roots were taken directly from plants grown at 24°C. Cold treatment of these plants resulted in a minimum three-fold increase in the number of morpho-

genic sectors produced (Table I.).

Regeneration frequencies were stable and reproducible during cold storage. As a result, we routinely used material from both RLD and Columbia plants stored in the cold room for up to four months.

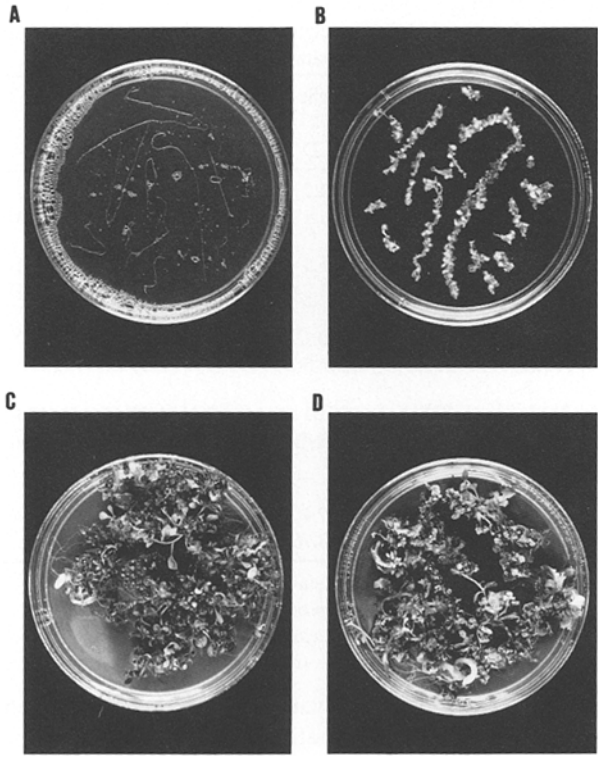

Fig. 1. Different stages of plant regeneration from root explants. **A**: 5 day-old root explants on ARM I medium. **B**: 10 day-old ARM IIr culture from the RLD genotype with adventitious polyembryonic regions. **C**: 10 day-old ARM III culture from the RLD genotype. **D**: Twenty day-old ARM III culture from the Columbia genotype. Previously these explants had been grown on ARM IIc containing 25 mg/I $AgNO_3$.

Induction of Embryogenesis

Most *in vitro* transformation procedures rely on separate stages of callus induction, shoot induction and, finally, root induction. For *Arabidopsis,* the entire process takes at least several months (Sheikholeslam and Weeks 1987, Valvekens *et al.* 1988, Koncz *et al.* 1990). More importantly, the length of time spent in tissue culture is associated with an increased frequency of aberrations in the plants finally regenerated. Minimizing the period of callus culture is expected to reduce such somaclonal variation (Bayliss 1980). An alternative strategy is to induce embryogenic growth at as early a stage as possible, since embryogenic cultures are known to be genetically more stable. Sporadic embryogenesis has been reported in their *Arabidopsis* transformation-regeneration system by Rédei *et al.* 1988, and Koncz *et al.* 1990.

In order to improve the efficiency of production of transgenic plants, we tried both of these approaches, a shortened culture induction phase (Valvekens *et al.* 1988) and a culture regime designed to induce embryogenesis.

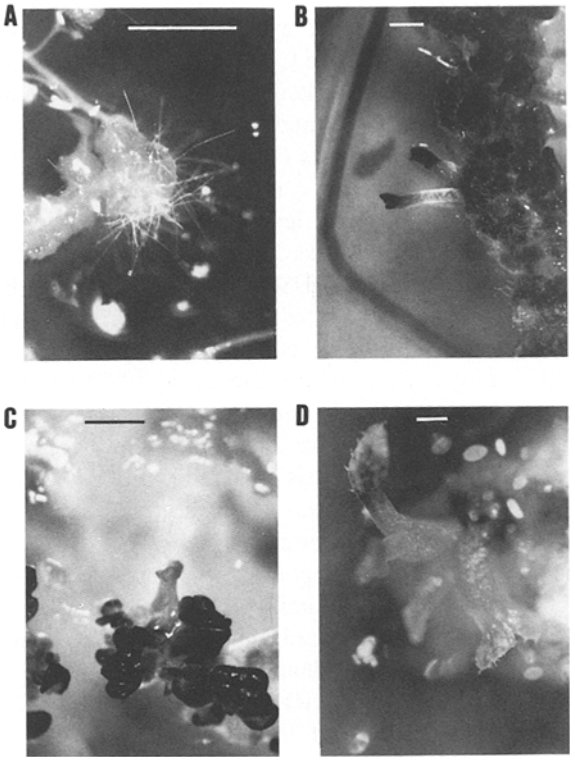

Fig. 2. Microphotographs show different stages of plant regeneration from root explants. **A**: 3 day-old ARM IIr culture with globular structures. **B**: Differentiation from the polyembryonic mass. **C**: Developing adventitious embryos. **D**: Fully differentiated plantlets. (The bar represents 1 mm in each photograph.)

Table I. Morphogenic response of RLD and Columbia root explants taken from plants grown at 5°C or 24°C. RLD or Columbia root explants were cultured on ARM I for 5 or 10 days, respectively and then transferred onto ARM IIr or ARM IIc. Morphogenic sectors were counted after seven days (for RLD) or 14 days (for Columbia).

Age of Seedlings [Weeks]	Morphogenic sectors per cm[a] plants grown at 5°C RLD	Columbia	plants grown at 27°C RLD	Columbia
1	-	-	-	-
2	-	-	16.1	2.6
3	25.6	3.2	25.6	3.2
4	-	-	>30	4.4
5	>30	14.0	>30	6.7
6	-	-	11[d]	5.1[d]
11	>30	16.8	-	-
15	>30	18.5	-	-
19	>30	16.4	-	-

[a] These appeared as compact green spots and microscopically as polymeristematic structures.
[b] Plants were transferred to 5°C at three weeks.
[c] Larger numbers were not enumerated because it was impossible to distinguish individual sectors
[d] Necrotic, senescent tissue.

Root explants incubated on ARM I for up to five days for RLD or up to ten days for Columbia, gave rise to regenerant plants principally via adventitious embryos. This short culture induction period on ARM I precluded

significant callus formation. In contrast, a longer culture induction period (>12 days) resulted in formation of green calli. These calli exhibited poor regeneration ability from both RLD and Columbia genotypes on ARM IIr or ARM IIc media. Plants, if any, produced at these longer times were obtained mainly via organogenesis rather than embryogenesis (see later in Table III).

We experimented with a number of protocols which employ moderate to high levels of 2,4-D to induce calli (Sheikholeslam and Weeks 1987, Valvekens *et al.* 1988, Schmidt and Willmitzer 1988) and, in our hands, these procedures did not lead to morphogenesis via adventitious somatic embryogenesis. Other recipes (Negrutiu *et al.* 1975, Lloyd *et al.* 1986, Rédei *et al.* 1988) use more balanced auxin/cytokinin ratios in the culture inducing medium. Using these, we observed a low frequency (1-5%) of morphogenesis via adventitious somatic embryogenesis from various explants in agreement with Rédei *et al.*(1988) and Koncz *et al.* (1990).

However, none of the regimes listed in Table II, or any other growth regulator combinations that we used, was able to provide regenerated Columbia plants at an efficiency that was more than 10% of the efficiency obtained for RLD on ARM IIr medium. To a large extent, the poor regeneration of the Columbia genotype appeared to be due to the action of endogenous ethylene. Silver nitrate inhibits ethylene action, and we previously found that the addition of $AgNO_3$ to culture media greatly improves regeneration of many dicot and monocot cultures (Purnhauser *et al.* 1987).

A morphogenic response as strong as that of RLD genotype could be seen in Columbia genotype with the inclusion of 25 mg/l $AgNO_3$ in the ARM IIc medium (Table II, Fig.1 D).

Three weeks later, the shoots (longer than 3-4 mm) could be transferred onto G-H medium for rooting and flowering. In order to get more plants, the morphogenic sectors were transferred onto ARM III medium. The simultaneous addition of GA_3 to this medium enhanced the elongation of the shoots. Using RLD, it was possible to obtain flowering plants in less than four weeks from the root explants. Approximately six weeks were required for the Columbia genotype.

Transformation of Root Explants.

The optimized regeneration procedures were used to obtain transgenic plants of RLD and Columbia after cocultivation transformation. In all experiments, transformation was carried out using the *pAL4404/-PMOGEN24* binary construction in Ach5 background. The *PMOGEN24* plasmid carries the P_{35S}-*hpt-nos3'* and the P_{nos}-*npt-ocsnos3'* cassettes for selection of transgenic plants on hygromycin and/or on kanamycin.

Generally, it is appropriate to carry out cocultivation before or during the culture induction phase since, after this time, the efficiency of transformation is reduced (Márton 1979). The optimal timing was determined by beginning cocultivation at various times and then scoring the efficiency of transformation by counting the number of green sectors produced on kanamycin containing media. In the untransformed cultures complete bleaching was seen. The data in Table III indicate that a four-day period of preconditioning was optimal for roots from RLD plants and that ten days were required in experiments using the Columbia genotype.

Table II. Efficiency of plant regeneration from RLD and Columbia genotypes of Arabidopsis thaliana under different growth regulator regimes during culture on ARM II media. Root explants were from RLD plants grown for three weeks at 24°C or from Columbia plants grown for three weeks at 24°C plus two weeks at 5°C. Explants were induced on ARM I medium for five days (RLD) or 10 days (Columbia) before being transferred to the various ARM II media. The number of shoots and fresh weight per cm of original root explant were measured 21 days later. Data are means ± std. error.

Growth Regulators [mg/l]	Fresh Weight [mg/cm]		Transferable Shoots [number/cm]	
	Columbia	RLD	Columbia	RLD
[a]0.2 NAA 0.4 IPA	135.4 ±5.49	211.2 ±41.15	0.74 ±0.12	16.29 ±1.23
0.2 NAA 2.0 IPAR	58.2 ±11.90	365.1 ±11.34	0.28 ±0.02	3.38 ±0.11
0.1 NAA 2.0 IPAR	50.2 ±2.56	240.1 ±14.50	1.35 ±0.07	8.89 ±0.70
[b]0.1 NAA 2.0 IPA 1.0 IPAR	53.8 ±1.98	221.1 ±18.41	1.44 ±0.11	7.33 ±0.39
As [b] above + 25.0 $AgNO_3$	56.1 ±8.56	157.4 ±8.76	>30[c]	>30[c]

[a] Named ARM IIr, used for regeneration of RLD cultures
[b] Named ARM IIc, used for regeneration of Columbia cultures
[c] Cultures were grown for two weeks on ARM IIc and then transferred onto ARM IIc+$AgNO_3$ medium. Cultures were scored 21 days later.

The number of green, kanamycin resistant sectors did not always correspond to high frequencies of transgenic plant recovery because with longer preincubation times the regeneration potential dramatically decreases (see above). For this reason we used a suboptimal preconditioning time (eight days as a routine), so that transformed sectors could maintain a higher, mainly embryogenic morphogenic potential.

Less than 20% of the shoots obtained from kanamycin selection were able to root under selective conditions. In the hygromycin selection experiments, the frequency of rooting was about 80% although the selection took longer (3-5 weeks). The presence or absence of 20 μM acetosyringone during cocultivation (Sheikholeslam and Weeks 1987), did not improve the transformation frequencies (data not shown). This may have been due to the high ratio of tissue to medium during cocultivation (0.5-1 g root material in 10 ml) which would result in high concentrations of natural inducers of the bacterial transformation machinery.

A final confirmation of transformation was sought by Southern blot analysis of DNA from individual regener-

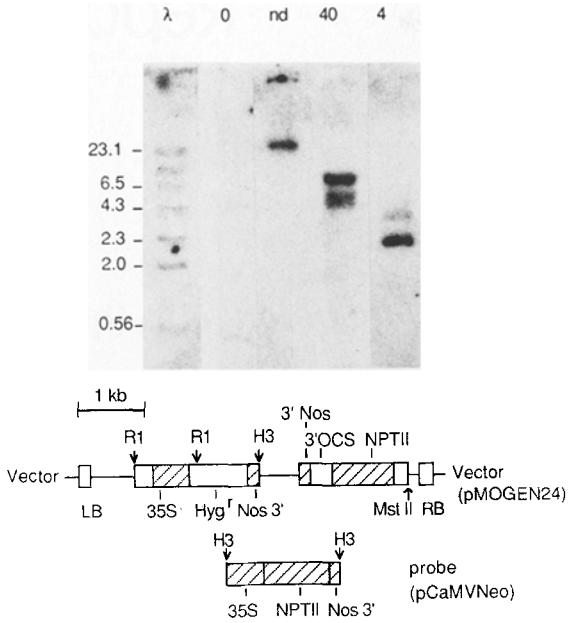

Fig. 3. Southern blot analysis of the putative transformants. After HindIII digestion of total DNA, the fragments were separated by agarose gel electrophoresis, blotted onto Zeta-Probe membrane and probed with the P_{35S}npt-nos3' fragment. **Lane 1**: phage lambda HindIII fragments as molecular weight markers; **Lane 2**: Untransformed control. **Lane 3**: Plant No. 40 prior to HindIII digestion. **Lanes 4 and 5**: Plants No. 40 and 4 digested with HindIII. Cartoon shows the structure of the vector and the probe.

ated plants. All the rooted plantlets tested contained sequences which hybridized to the T-DNA probes (Fig. 3). The unique *Hin*dIII site on the *pMOGEN24* vector allowed us to discriminate between integrated and non-integrated forms of the vector DNA. After *Hin*dIII digestion of the total plant DNA, the Southern blot would be expected to give a single 12 kbp fragment for a non-integrated vector molecule when probed with P_{35S}-*npt-nos3'*. The presence of two or more fragments of less than 10 kbp in plants 4 and 40 (Fig. 3) indicated integration of the T-DNA into the plant genome. About half of the plants analyzed showed a pattern on Southern analysis that was consistent with the presence of a single copy of T-DNA, which was in line with the 3:1 segregation for resistance in their selfed progeny.

As a conclusion, based on the results presented above, a step-by-step protocol can be given for facile transformation of Arabidopsis root explants. **Obtain root explants** from axenically germinated seedlings which were grown for at least 3 weeks at 24°C and stored at 4°C up to four months. (For the Columbia genotype at least two weeks cold treatment was used). **Preincubate** full length roots in bulk on ARM I medium for three days if RLD, and at least five but not longer than eight days if Columbia genotype is used. Collect the roots, wound and coculture for two days as described. **After** removal of the bacterial mass, postincubate the roots in bulk on ARM I with claforan and carbenicillin for two days. **Spread** the individual roots on the surface of ARM IIc or IIr media with kanamycin and/or hygromycin as described and culture for two weeks. In RLD cultures the putative transgenic shoots can be scored directly and subcultured on selective ARM III medium and rooted. **Columbia cultures** should be subcultured on ARM IIc medium containing 25 mg/l silver nitrate for another two weeks before the putative transformants are scored. If the shoot development is not appropriate on ARM III medium, it can be supplemented by 1 mg/l GA_3.

Table III. Effect of the different preconditioning periods on ARM I medium on the frequency of kanamycin (km) resistant sectors after cocultivation with Agrobacterium tumefaciens, LBA4404 (pM0GEN24).

Preconditioning[a] on ARM I [days]	Number of inocula[b] plated RLD	Columbia	km resistant RLD	Columbia	Expressed as % RLD	Columbia
0	20	30	4	0	20.0	0
1	22	19	10	0	45.4	0
2	17	27	9	1	52.9	3.7
3	24	32	11	0	45.8	0
4	35	29	31	2	88.5	6.9
5	28	26	22	9	78.5	19.3
10	32	31	23	9	71.8c	29.0c
15	25	30	21	8	80.4c	26.6c

[a] Preconditioning was always followed by 2 day cocultivation in MS-H medium then by 2 days of postincubation on ARM I medium containing 200 mg/l each of carbenicillin and claforan to eliminate bacterial growth.
[b] Inoculum represents 1 cm of original root explant.
[c] Green calli giving poor regeneration.

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