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Specialized vectors for gene tagging and expression studies

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Introduction

A genetic approach for the functional identification of genes involves mutagenizing the genome with a known, unique DNA sequence that provides both phenotypic and molecular markers for the isolation and mapping of gene mutations, and the cloning of corresponding genes. The efficiency of a DNA tag as a mutagen is primarily determined by the frequency and randomness by which it can be introduced into the genome of a target organism, and to a lesser extent by its physical or genetic properties that may be modified by genetic engineering. In contrast to base pair exchanges and deletions induced by chemical and physical mutagens that may not result in a complete loss of gene function, insertional mutagens are believed to cause only null mutations by a structural interruption of genes. However, this view is not entirely correct, because insertions in promoters are known either to positively or negatively affect gene expression, whereas insertions in coding regions may also result in gene fusions encoding truncated, but still functional, proteins. In addition, mutations induced by insertion elements frequently cause polar effects enhancing or reducing the transcription of genes located in the vicinity of insertions. It is therefore not by chance that gene tagging by insertion elements is one of the most powerful methods in the molecular analysis of gene expression [1].

Seminal work on the application of insertion elements to create *in situ* gene fusions, and thus measure gene expression in living cells, was reported by Casabadan and Cohen [2, 3]. The idea to link promoterless reporter genes to the termini of insertion elements, and thereby generate transcriptional or translational fusions between target and reporter genes, was adapted to many bacterial phages and transposons [4, 5], as well as to eukaryotic transposable elements such as the P-element of *Drosophila* [6] and retrotransposons (or retroviruses) of yeast and mammals [7, 8]. Long terminal repeats of many eukaryotic insertion elements, however, prevent the application of direct gene fusion technology. This problem was overcome by an elegant technical modifi-

cation, first applied with the P-element. A reporter gene driven by a TATA-box minimal promoter was linked to the end of a P-element, and was used to detect transcriptional enhancer and silencer elements by tagging of developmentally regulated genes in *Drosophila* [9, 10]. To activate gene expression in a regulated fashion, transposons carrying a strong *tac* promoter at their ends were developed for gene tagging in bacteria [11, 12].

Although transposable elements were first discovered in plants, their application to gene fusion technology is only very recent [13]. Development of gene tagging techniques in plants required the establishment of a reliable transformation technique that was achieved by application of the T-DNA of *Agrobacterium* Ti and Ri plasmids as a wide host range plant vector. Analysis of the mechanism underlying the T-DNA transfer process demonstrated that any DNA fragment linked to specific 25 bp border repeats of the T-DNA can be transferred from *Agrobacterium* into plants when Ti or Ri plasmid encoded virulence gene functions are provided in either *cis* or *trans*. T-DNA was shown to be stably and randomly integrated in the plant nuclear genome [14, 15]. Therefore, with the availability of chimeric plant selectable markers, T-DNA was exploited as a plant insertion element.

To detect T-DNA insertions in plant genes by the gene fusion technique, a promoterless *aph*(3′) II gene of Tn*5*, encoding a neomycin phosphotransferase (NPT II), was linked to the right end of the T-DNA and used for selection of gene fusions conferring kanamycin resistance to transgenic explants of *Nicotiana* species [16–18]. However, direct selection for active gene fusions was a failure, because it resulted in the accumulation of aberrant T-DNA inserts at a high copy number [19]. This, together with the difficulty of genetic analysis in *Nicotiana*, led to the improvement of vector design, and to the use of *Arabidopsis thaliana* as a model plant with a small genome and excellent genetics [19–22].

T-DNA gene fusion vectors used today are equipped with suitable plant selectable markers that confer resistance against either antibiotics or herbicides, and with bacterial plasmids and marker genes that facilitate the reisolation of inserts from the plant genome by plasmid rescue or alternative cloning techniques [23]. Derivatives of a T-DNA vector described in this chapter provide three different promoterless reporter genes linked to the right T-DNA border for identification of plant gene fusions. Gene tagging by these vectors allows the use of a variety of reporter enzyme assays by which the activity of *in situ* gene fusions can be detected either *in vivo* or *in vitro*. By insertion of minimal TATA-box promoters between the T-DNA border and these reporter genes, the tagging vectors can be modified for the detection of enhancers as described [24, 25], in analogy to the P-element system [10]. An exchange of strong promoters, enhancers or silencers for the reporter genes at the T-DNA borders provides vectors for activation or repression of gene expression by T-DNA-tagging. In analogy to insertional mutagenesis by transposons carrying a *tac* promoter in bacteria [11], this approach was successfully used to identify regulatory genes in *Nicotiana* [26]. Once genes are identified by T-DNA tagging

and rescued in *Escherichia coli*, their transcriptional regulatory elements can be further studied using promoter and enhancer test vectors described below, that offer NPT II, β-glucuronidase (GUS), and bacterial luciferase (LuxF) reporters for the analysis of gene expression in transgenic plants.

Procedures

The use of PCV Agrobacterium *binary vectors*

1. Plant cloning vectors (PCVs, Figs. 1, 3 and 4) consist of two functional units: a conditional mini-RK2 replicon and the T-DNA. Between the left and right 25 bp borders (L_B and R_B, respectively) the T-DNA carries plant selectable markers, reporter genes, cloning sites and a segment of plasmid pBR322 with a ColE1 replication origin (ori_{pBR}), as well as a β-lactamase gene providing ampicillin and carbenicillin resistance (Ap^R/Cb^R) for selection in *E. coli* and *Agrobacterium*. The mini-RK2 segment contains both the vegetative (*oriV*) and conjugational (*oriT*) DNA replication origins of plasmid RK2. These conditional *ori* functions are active only when *trans*-acting helper functions for RK2 replication and conjugation (i.e. *trfa* and *Tra*) are present in *E. coli* and *Agrobacterium* [27]. Therefore, PCVs can be used as simple ColE1-derived vectors in standard *E. coli* hosts without a problem of instability caused by duplication of replication origins. In contrast to other binary vectors (e.g. pBIN series), PCVs do not express any *kil* gene function from RK2 that could cause cell death during storage of bacteria.
2. PCVs can be transferred from *E. coli* to *Agrobacterium* by
   - low frequency mobilization of the pBR replicon using helper plasmids GJ23 and R64*drd*11 [28], or by
   - high frequency mobilization of the RK2 replicon using RK2 helper functions carried by plasmids (such as pRK2013 or pCT153.1 [29, 30]), or by the chromosome of *E. coli* helper strains (such as S17–1 and SM10, [31]), and by
   - transformation [32] or electroporation [33].
3. *Agrobacterium* strain GV3101 (pPM90RK) is a standard host for PCVs. This strain harbors a C58C1 chromosomal background marked by a rifampicin resistance (Rif^R) mutation, and carries pMP90RK, a helper Ti plasmid encoding virulence functions for T-DNA transfer from *Agrobacterium* to plant cells. pMP90RK is a 'disarmed' derivative of pTiC58

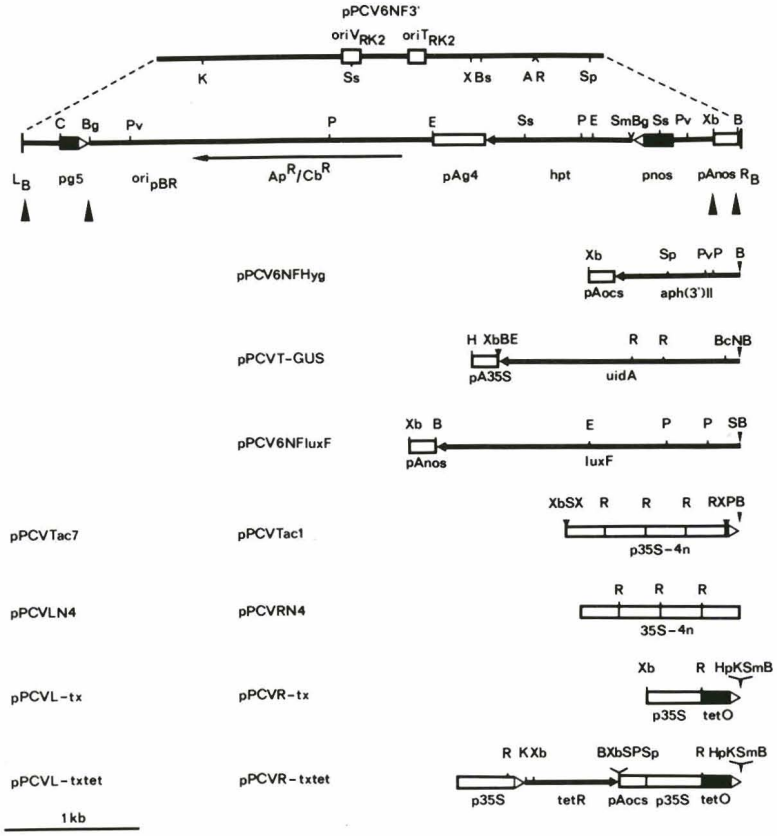

Fig. 1. PCV vectors for isolation of plant gene-reporter gene fusions and regulation of plant gene expression by T-DNA tagging. The upper lane shows the mini-RK2 segment of PCVs that carries the vegetative (*oriV*) and conjugational (*oriT*) DNA replication origins of RK2. In the second lane the T-DNA is depicted that is flanked by the left (L_B) and right (R_B) 25 bp border repeats, and carries a ColE1 replication origin (ori_{pBR}) and a β-lactamase gene encoding ampicillin and carbenicillin resistance (Ap^R/Cb^R) from pBR322. Lanes below the physical map of plasmid pPCV6NF3′ (a precursor of T-DNA gene fusion vectors), show reporter gene or promoter constructs that were inserted into restriction endonuclease cleavage sites of pPCV6NF3′ marked by vertical black arrows. For example, pCV6NFHyg thus carries an *aph*(3′) II, neomycin phosphotransferase gene from transposon Tn*5* linked to the polyadenylation sequence of the octopine synthase gene (pA_{ocs}) cloned into *Xba*I and *Bam* HI sites of plasmid pPCV6NF3′. In the case of vectors pPCVTac7, pPCVLN4, pPCVL-tx and pPCVL-txtet, the promoter and enhancer segments, shown to the right for pPCVTAc1, pPCVRN4, pPCVR-tx and pPCVR-txtet, were inserted close to the left T-DNA border in opposite orientation (i.e. such that the 3′-ends of these promoters face the left 25 bp border repeat). *Abbreviations*: *hpt*, hygromycin phosphotransferase [19], *aph*(3′) II, aminoglycoside (neomycin) phosphotransferase [19]; *uid*A, β-glucuronidase (from pRAJ275, [45]); *lux*F, fusion bacterial luciferase A&B (from pLX702fab, [57]) genes. p35S-4n, a Cauliflower Mosaic Virus (CaMV) 35S promoter carrying 4 repeats of the −90 to −440 enhancer domain [26], 35S-4n, the latter CaMV35S enhancer repeats without the +1 to −90 promoter domain; tetO, 3 repeats of tetracycline operator sequences [48]; tetR, a tetracycline repressor gene from transposon Tn*10* [48], pg5, the promoter of T_L-DNA gene 5 [27]. pA, polyadenylation sequences from the nopaline synthase gene (pAnos), from the octopine synthase gene (pAocs), from gene 4 of the T_L-DNA (pAg4), and from the 35S RNA gene of CaMV (pA35S). Restriction endonuclease cleavage sites: A, *Apa*I; B, *Bam* HI; Bg, *Bgl* II, Bc, *Bcl* I; C, *Cla* I; E, *Eco* RI; H, *Hin*d III; Hp, *Hpa* I; K, *Kpn*I; N, *Nco* I; P, *Pst* I; Pv, *Pvu* II; R, *Eco* RV; S, *Sal* I; Sp, *Sph* I; Sm, *Sma* I; Ss, *Sst* II; X, *Xho* I; Xb, *Xba* I.

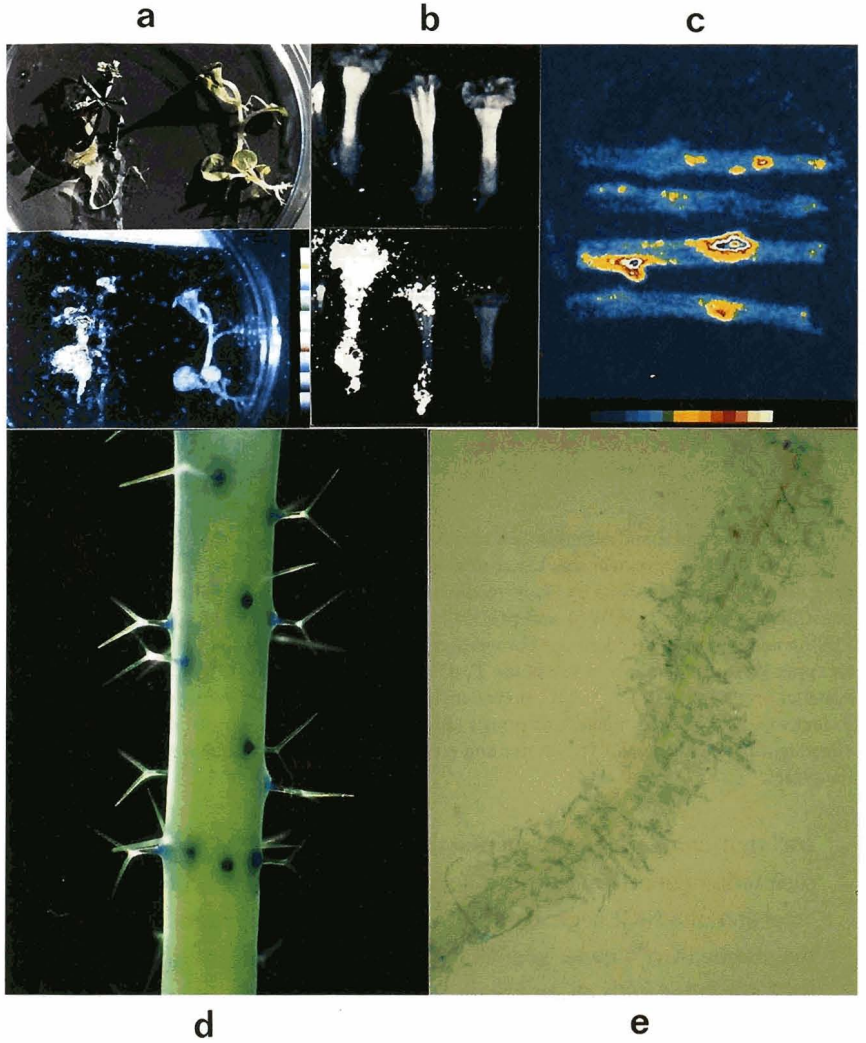

Fig. 2. Screening for the activity of luciferase and β-glucuronidase gene fusions in transgenic *Nicotiana* and *Arabidopsis* plants. (a) An example for visualization of luciferase gene expression in *Arabidopsis*. Upper section: visual image of a transgenic (left) and a wild type (right) *Arabidopsis* plant carrying *lux*A and *luxB* genes under the control of 1′ and 2′ promoters of the mannopine synthase (*mas*) gene. Lower section: an overlay of luminescent image of luciferase mediated light emission on the visual image. (b) Upper section: visual image of flowers from transgenic (left and middle) and wild type (right) tobacco plants. Lower section: corresponding image of luciferase light emission in transgenic flowers. (c) Activation of *mas* promoter luciferase gene fusion in axillary buds of decapitated tobacco stem sections (for details see [46]). (The pictures were obtained as a courtesy from WHR Langridge and AA Szalay, University of Alberta, Edmonton, Canada.) (d) Histological staining for β-glucuronidase activity in stem trichomes of a pPCVT-GUS transformed *Arabidopis* plant. (e) Detection of promoter activity using pPCV812 in root hairs of a transgenic *Nicotiana* plant.

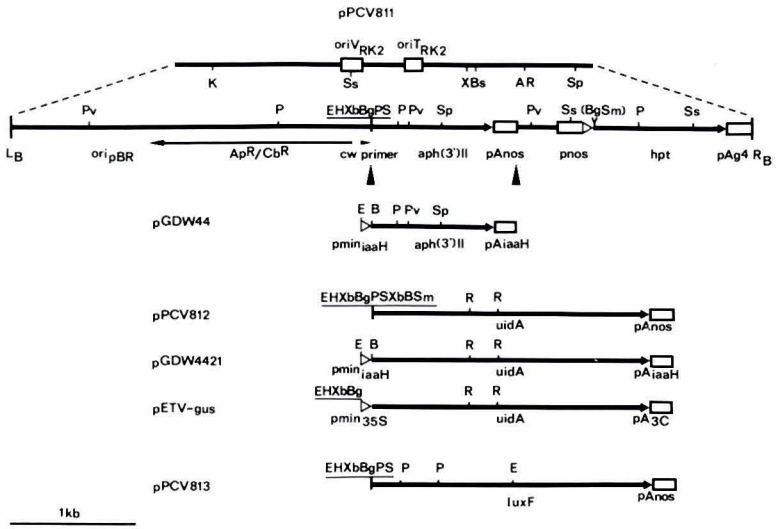

Fig. 3. PCV promoter and enhancer test vectors. The upper lane, as in Fig. 1 shows the mini-RK2 segment of PCV vectors, whereas the second lane displays the T-DNA. The *aph*(3′) II reporter gene of pCV811 [23] marked by black vertical arrows was replaced by alternative reporter gene constructs in vectors pGDW44 and pGDW4421 [54]; pPCV812 and 813 [23]; and pETV-gus [55] depicted in the other lanes. Abbreviations are identical with those listed in Fig. 1, except for: pmin*iaa*H, minimal promoter of the T_L-DNA gene 2 [54]; pmin35S, CaMV35S TATA-box minimal promoter [55], and pA3C, polyadenylation sequence of pea *rbcS-3C* gene. CW primer: a clockwise pBR322 oligonucleotide primer (Biolabs) that hybridizes upstream of the *Eco* RI site allowing direct sequencing of promoter and enhancer constructs in double-stranded PCV DNA templates.

tra^c that carries a complete deletion of wild type T-DNA, a gentamycin resistance gene (Gm^R) in the nopaline catabolism operon (*Noc*), and an insertion of a RK2 segment from pRK2013 (29) marked by a kanamycin resistance (Km^R) gene. pMP90RK acquired the conjugative properties of RK2 together with all functions required for replication and mobilization of PCV-type mini-RK2 plasmids. When necessary, PCV constructs can therefore be conjugated back from *Agrobacterium* to *E. coli* to test their structural stability.

4. To use PCVs in agrobacteria with different host-ranges, or in combination with various Ti or Ri plasmids, the RK2 helper functions can be integrated into the chromosome of any *Agrobacterium* strain using illegitimate recombination mediated by an *IS21* insertion element of nonreplicative RK2 derivatives (e.g. pCT153.1 [27, 30, 34]). Similarly, PCV plasmids without a ColE1 replication origin can be maintained as autonomous mini-RK2 replicons in *E. coli* strains carrying chromosomally integrated RK2 helper functions [31]. A combination of PCV mini-RK2 replicons

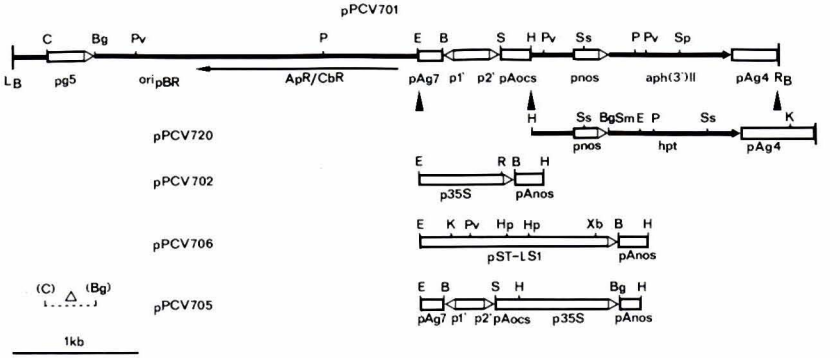

Fig. 4. PCV expression vectors. Structure of the full-lenght T-DNA of vector pPCV701 is shown in the upper lane. Promoter and gene constructs depicted in the other lanes were cloned to restriction endonuclease cleavage sites marked by vertical black arrows, by replacing segments of the pPCV701 T-DNA. p1′ and p2′, bidirectional promoters of the T_R-DNA mannopine synthase (*mas*) genes in plasmids pPCV701 and pPCV720 [23, 56]; pST-LS1, a light regulated promoter of the potato ST-LS1 gene in plasmid pPCV706 [20]; pAg7, polyadenylation signal of T_L-DNA gene 7. A triangle between (C) and (Bg) indicates a deletion of the T_L-DNA gene 5 promoter in expression vector pPCV705. Other abbreviations are identical to those listed in Fig. 1. An example for use of these vectors is described in [20].

with bacterial transposons and phages can therefore be exploited for construction of:

- conditionally replicative transposons with T-DNA functions, to create reporter gene fusions by *in vivo* mutagenesis of plant genes cloned in *E. coli*, followed by transfer of these gene fusions via *Agrobacterium* into plants for functional analysis [35, 36], and
- λ phage vectors with T-DNA functions that behave as conjugative RK2 replicons (e.g. in a λcI^{ts} lysogenic *E. coli* strain carrying pRK2013), and allow the transfer of cDNA libraries from *E. coli* to *Agrobacterium*, and later to plants.

5. In addition, PCV vectors can easily be converted to cointegrate type vectors using pMP90, a derivative of pMP90RK without RK2 helper functions [27], and double cross-over recombination with the Gm^R gene located in the *Noc* region of pMP90.
6. PCVs, as other *Agrobacterium* mini-RK2 binary vectors, lack plasmid partitioning functions providing stable inheritance. For stable maintainance, *Agrobacterium* strains carrying PCVs should always be grown in the presence of antibiotic selection. Induction of *IS21* by shock freezing may cause deletions in the helper Ti plasmid, pMP90RK. Therefore, conjugational transfer of PCVs is recommended rather than transformation and electroporation techniques. Nonetheless, once a PCV

plasmid is present in *Agrobacterium* strain GV3101(pMP90RK), a selection for the antibiotic resistance marker of PCV ultimately provides selection for the maintainance of RK2 helper functions, too. In case the RK2 helper *Agrobacterium* host is lost (e.g. due to shock freezing of glycerol stocks), it can always be recovered from PCV containing strains, that can easily be cured of their PCV by growing them in the absence of antibiotic selection.

Strain construction, conjugation, storage of bacteria

Agrobacterium strains are grown in YEB-medium (for 1 l: 5 g beef extract, 1 g yeast extract, 5 g peptone, 5 g sucrose + 2% agar for plates and 2 ml 1M $MgCl_2$ after sterilization), *E. coli* strains are cultured in LB [37].

1. Test *Agrobacterium* strain GV3101 (pMP90RK) by streaking on YEB plates containing either 100 µg/ml rifampicin (Rif_{100}) and 25 µg/ml kanamycin (Km_{25}), or Rif_{100} and 25 µg/ml gentamycin (Gm_{25}); then grow in YEB at 28 °C to late logarithmic phase. Purify *E. coli* donor strain S17–1 [36] on LB plates containing 100 µg/ml streptomycin (Sm_{100}). Transform PCV plasmids into S17-1, as described [37]. Purify PCV transformants on LB plates containing 100 µg/ml ampicillin. Inoculate and grow S17–1 (PCV) strains in LB at 37 °C to late logarithmic phase. *Note*: for most cloning purposes S17–1 is a suitable host. When larger plant DNA fragments are cloned into PCVs, it is advisable to carry out the cloning in *E. coli* DH5α, or in other standard *recA* hosts, and then transform PCV constructs to S17–1 to assay for their stability.
2. Dilute both *E. coli* S17–1 (PCV) donor and *Agrobacterium* GV3101(pMP90RK) recipient to OD_{550}: 0.5 (approximately 1×10^8 cfu/ml) and incubate them at 28 °C for a further 10 min. Mix equal volumes of donor and recipient cells in a test tube. Place droplets of 100–200 µl conjugation mix, and drops of donor and recipient on a YEB plate, and allow to dry. Incubate the conjugation plates at 28 °C for 24 h. Take a loop of bacteria from the conjugation spots and streak out to single colonies on YEB Rif_{100} plates containing 100 µg/ml carbenicillin (Cb_{100}). Alternatively: scrape off bacteria from the conjugation spots in 5 ml YEB, and plate a series of dilutions on YEB Rif_{100} Cb_{100} plates. Conjugation frequencies for PCV plasmids of max. 20 kb should be in a range of 1–10%. Transconjugants appearing within 3 days are purified, then grown in 10 ml liquid YEB Cb_{100}, pelleted by centrifugation, resuspended in 1 ml YEB, mixed with 1 ml 87% glycerol, and stored at −70 °C. Alternatively: *Agrobacterium* stocks can be stored indefinitely on YEB Cb_{100} plates, or in stabs by monthly subculturing.
3. *Note*: This protocol is applicable for mobilization of PCV plasmids from all RK2 donor *E. coli* strains to any *Agrobacterium* recipient carrying RK2 helper functions, as well as for conjugation of the pMP90RK helper Ti plasmid to other *Agrobacterium* hosts. New *Agrobacterium* helper strains can analogously be constructed by mobilization of pCT153.1 from *E. coli* [30] to different *Agrobacterium* strains. Spontaneous Rif^R mutants can be isolated in *Agrobacterium* at a frequency of about 1×10^{-8}, whereas Km^R transconjugants carrying a chromosomally integrated copy of pCT153.1 are obtained at frequencies between 10^{-7} to 10^{-8} transconjugants/recipient. To recover PCV constructs from *Agrobacterium* in *E. coli*, perform conjugation between *Agrobacterium* GV3101 (pMP90RK) (PCV) donors and standard *E. coli* hosts, as described above. Plate dilutions from the conjugation mix on LB plates containing 100 µg/ml ampicillin, and incubate the plates overnight at 37 °C. *Agrobacterium* grows poorly, if at all at 37 °C. This provides a good selection for *E. coli* transconjugants harboring PCVs.

Plant transformation: the Arabidopsis *model*

PCV vectors in *Agrobacterium* GV3101 (pMP90RK) were successfully used for high frequency transformation of many plant species, such as *Nicotiana, Medicago, Brassica, Solanum, Craterostigma, Fragaria, Lycopersicum, Populus, Arabidopsis*, etc. Because of the significant impact of T-DNA tagging upon *Arabidopsis* genetics, a protocol applicable for the transformation of different *Arabidopsis* ecotypes is given below.

a. Arabidopsis *tissue culture media*

MSAR medium is a modified MS medium (38) for *Arabidopsis* that can simply be prepared from stocks listed in Table 1.

To prepare 1 l MSAR medium measure together the following amounts of stock solutions: 50 ml Macro, 1 ml Micro, 5 ml Fe-EDTA, 5.8 ml $CaCl_2$, 2.2 ml KI, 2 ml vitamins, and 30 g sucrose. Adjust the pH to 5.8 by 0.5 M KOH, add 2.2 g phytagel (Sigma, or gelrite from Kelco Co.), and autoclave the medium for 15 min. *Note*: phytagel or gelrite is essential for tissue

Table 1. MSAR stock solutions

1. *Macro*	(for 1 l):	20 g NH_4NO_3; 40 g KNO_3; 7.4 g $MgSO_4 \cdot 7H_2O$; 3.4 g KH_2PO_4; 2.0 g $Ca(H_2PO_4)_2 \cdot H_2O$
2. *Micro*	(for 1 l):	6.2 g H_3BO_3; 16.9 g $MnSO_4 \cdot H_2O$; 8.6 g $ZnSO_4 \cdot 7H_2O$; 0.25 g $Na_2MoO_4 \cdot 2H_2O$; 0.025 g $CuSO_4 \cdot 5H_2O$; 0.025 g $CoCl_2 \cdot 6H_2O$
3. *Fe-EDTA*	(for 1 l):	dissolve separately: 5.56 g $FeSO_4 \cdot 7H_2O$, and 7.46 g $Na_2EDTA \cdot 2H_2O$; mix the two solutions, adjust the volume to 1 l
4. *$CaCl_2$*	(for 1 l):	7.5 g $CaCl_2 \cdot 2H_2O$
5. *KI*	(for 1 l):	375 mg KI
6. *Vitamins*	(for 1l):	50 g *myo*-inositol; 2.5 g thiamine · HCl; 0.5 g nicotinic acid, 0.2 g pyridoxine · HCl

culture and regeneration of *Arabidopsis*, because agar media give a very poor response.

Media used for the transformation and regeneration cycle of *Arabidopsis* are summarized in Table 2.

b. Arabidopsis *root culture*

1. Place 10 mg (about 500) seeds in an Eppendorf centrifuge tube and shake with 1 ml sterilization solution (5% $Ca(OCl)_2$, 0.02% Triton X-100) for 15 min. Pellet the seeds by brief centrifugation, remove the calcium hypochlorite solution, rinse the seeds with 4 × 1 ml sterile H_2O, and dry the tubes in a flow-hood overnight. Distrupt seed clumps with a sterile toothpick, and sow seeds on SG plates.
2. When the first rosette leaves appear transfer 1–3 plantlets into Erlenmeyer flasks containing 15 ml MS liquid medium [38]. Place the flasks on a slowly rotating illuminated shaker (50 rpm). Depending on the light conditions and temperature, within 14–20 d the volume of liquid medium will be filled with roots. For transformation harvest roots only from plants that do not show senescence.

c. Tissue culture transformation of Arabidopsis *by* Agrobacterium

1. Grow *Agrobacterium* strains carrying PCVs in YEB Cb_{100} medium to OD_{550}: 1.5, pellet the cells by centrifugation (5 K, 15 min), then resus-

Table 2. MSAR media for transformation and regeneration of *Arabidopsis*

1. Medium for seed germination (SG):	MSAR, but with 1/2 concentration of Macro stock and 0.5% sucrose
2. Auxin conditioning medium (MSARI):	MSAR supplemented with mg/l: 2.0 IAA, 0.5 2,4-D, 0.2 kinetin, 0.2 IPAR
3. High cytokinin medium (MSARII):	MSAR supplemented with mg/l: 2.0 IPAR, 0.05 NAA
4. Shoot elongation medium (SE):	MSAR supplemented with mg/l: 0.2 BAP, 0.1 IBA, 0.05 NAA

Abbreviations: IAA, indole-3-acetic acid; NAA, α-naphtaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; IBA, indole-3-butyric acid; kinetin, 6-furfurylaminopurine; IPAR, N^6-[2-isopentenyl]-adenosine; BAP, 6-benzylaminopurine.

pend them in MSARI liquid medium at OD_{550}: 0.5, and pour 50 to 80 ml suspension into large Petri dishes.
2. Take *Arabidopsis* plants from the Erlenmeyer flasks, remove their roots, place the roots into the *Agrobacterium* suspension, cut them into small pieces, and incubate for at least 30 min with bacteria. Following incubation remove the bacterial suspension from the Petri dishes with a pipette, collect the root segments and transfer them to sterile Erlenmeyer flasks with 40 ml MSARI medium. Culture the roots for 36 to 48 h on a shaker, then replace the medium by MSARI (without phytagel) containing 300 mg/l claforan (cefotaxim) and 500 mg/l tricarcillin/ clavulanic acid mixture (15 : 1, Duchefa Co., The Netherlands). (When tricarcillin is not available use 500 mg/l claforan). Subculture root cultures 3 times after 4 d intervals. *Note*: The length of time necessary for auxin conditioning varies between ecotypes. A few hours is sufficient for RLD, whereas Columbia and Landsberg require several days. Prolongation of the time of auxin treatment results in a gradual decrease in the efficienty of induction of embryogenesis and shoot formation by cytokinin treatment.
3. After 14 d transfer the roots from MSARI liquid culture to a large Petri dish, remove excess medium, and spread well-separated root explants on MSARII plates containing claforan (300 mg/l) and tricarcillin/clavulanic acid (500 mg/l). Depending on the type of selectable marker encoded by the T-DNA, the medium is supplemented with either 15 mg/l hygromycin

(Calbiochem) or 100 mg/l kanamycin sulfate (Sigma). Subculture the explants for 5 times after 14 d intervals using the same medium. In the last two transfers reduce the concentration of claforan to 200 mg/l, and omit tricarcillin/clavulanic acid and kanamycin, if used, from the medium. *Note*: In contrast to hygromycin, kanamycin inhibits shoot regeneration in *Arabidopsis*. During the first three week period nontransformed tissues die and green transgenic calli appear on the plates. Auxin conditioning followed by cytokinin treatment of root explants induces the development of embryo-like structures that quickly differentiate to shoots. Thus, within 4 to 8 weeks on average 60% of calli are converted to plantlets without roots (root development is inhibited by cytokinin). In subcultures particular care should be taken to separate shoots emerging from these embryogenic calli, as well as to separate all transformed calli from the dead explants. Once shoots reach a size of 4–8 mm, they are transferred for 3–4 d to SE medium in glass jars. Elongated shoots are then placed in test tubes (20 cm × 2.5 cm diameter) containing 20 ml SG medium, and the tubes are closed by loose cotton-wool plugs. Within days these plants start blooming, and provided the humidity in the tubes did not prevent the release of pollens from the anthers, set seeds. By starting from roots grown in a single Erlenmeyer flask yielding explants for 10 MSARII plates, the transformation procedure results in an average of 20 to 100 transgenic calli per Petri dish, and 2,000 to 10,000 transformants for 10 flasks.

T-DNA insertional mutagenesis techniques

Application of the T-DNA insertion mutagenesis is dependent on the efficiency of *Agrobacterium*-mediated transformation of a given plant species. In spite of continuous improvement of *in vitro* and *in planta* transformation methods [21, 22], the generation of T-DNA insertion mutants is a labour intensive approach. The amount of labor required is determined by the size of the target plant genome, the level to which the genome is being saturated by T-DNA inserts, and the simplicity of mutant selection or screening methods. Assuming a genome size of 100 Mb and a 1 kb resolution, the generation, maintainance and screening of offspring from over 10^5 individual transformants is required for tagging a given gene at a 95% probability in *Arabidopsis*. Although this number can easily be reached, saturation T-DNA mutagenesis with plants will always remain a heroic action. Nonetheless,

protoplast, leaf-disc, or root transformation offer a 'Petri dish scale' approach and selection methods, similar to those used in bacterial and yeast genetics.

Selection and screening for T-DNA-induced reporter gene fusions

Analysis of the frequency of T-DNA-induced reporter gene fusions indicated that T-DNA inserts are preferentially integrated into chromosomal loci that are potentially transcribed [19]. Studies of chromosomal target sites of T-DNA insertions have demonstrated that T-DNA integration occurs at various target sequences by illegitimate recombination [39, 40]. Using T-DNAs with suitable antibiotic resistance markers, it was also determined that the copy number of intact T-DNA inserts is 1 or 2 in 2/3 of all transformants. A screening for the expression of a promoterless *aph*(3′) II reporter gene showed that about 20 to 30% of T-DNA inserts generate plant gene fusions that are expressed in calli, as well as in various plant organs [19]. From these data it is predictable that in a population of 10,000, about 800 to 1000 transformants should carry single copy T-DNA inserts as linked plant gene-reporter gene fusions. Depending on the activity of plant promoters and fusion reporter proteins, a large proportion of these gene fusions can be identified by selection or screening in tissue culture. The earlier the selection or screening for active gene fusions, the lower is the need for labour and material. Gene fusion vectors pPCV6NFHyg, pPCVT-GUS and pPCV6NFluxF offer three different reporter genes for such selection and screening experiments (Fig. 1, for description of alternative vectors see [41, 42].

1. Using the *aph*(3′) II reporter gene in pPCV6NFHyg, gene fusions can be selected by plating regenerated protoplasts, microcalli, roots, leaf-discs, stems, shoots or seeds on media containing different concentrations of kanamycin and plant growth factors. A semiquantitative gel-assay for neomycin phosphotransferase provides a simple means for further analysis of the activity of *aph*(3′) II gene fusions as described [43]. Specific oligonucleotide primers hybridizing to the coding strand of *aph*(3′) II sequence allow quantitative measurement of fusion mRNAs by RACE PCR [44]. The latter method is generally applicable to all reporter gene fusions.
2. An *uid*A reporter gene in pPCVT-GUS facilitates screening for gene fusions throughout plant regeneration and development using histological

staining and sensitive fluorimetric assays for β-glucuronidase activity (Fig. 2, for methods see: [45]).

3. To screen large populations of T-DNA transformants for active gene fusions using a nondestructive reporter enzyme assay, a bacterial luciferase reporter gene is used in pPCV6NFluxF. *In vivo* low light emission by the bacterial luciferase can be followed in plants by real time video imaging using recent improvements of detection systems [46]. Although methods allowing an easy sorting of protoplasts or cells expressing the *luxF* gene are still under development, progress in video imaging technology, and the use of different light emitting reporters (e.g. firefly luciferases and aequorin) and luminescent substrates offer many novel possibilities for mutant screening. A simple, fast and quantitative *in vitro* luminometric assay is described below.

Luminometric assay of bacterial luciferase in plant exctracts

Stock solutions in 50 mM Na-phosphate (pH 7.0), stored at −20 °C:
- 100 mM $NADH^+$ (Boehringer).
- 5 mM FMN (Sigma).
- 20 U/ml $NADH^+$/FMN oxidoreductase (Boehringer, in 50 mM Na-phosphate (pH 7.0), 0.2% bovine serum albumin (BSA), 50% glycerol buffer).

Extraction buffer: 50 mM Na-phosphate (pH 7.0), 1 µg/ml leupeptin and α_2-macroglobulin (Boehringer).

Assay buffer: 100 µM FMN in 50 mM Na-phosphate (pH 7.0), 0.2% BSA.

Substrate: n-decanal (Sigma): dilute n-decanal in ethanol to 10%, inject 1 ml 10% n-decanal to 99 ml 50 mM Na-phosphate (pH 7.0) to obtain a fine emulsion.

Assay

1. Extract 1 to 100 mg of plant tissues with 100 µl extraction buffer in Eppendorf tubes on ice (e.g. by homogenization with sterile quartz), take aliquotes for determination of protein concentration.
2. Pipette into a luminometer (e.g. Berthold Biolumat LB9500C) vial
   - 500 µl extraction buffer.
   - x µl plant extract.

   - 10 µl $NADH^+$/FMN oxidoreductase.
   - 10 µl 100 mM $NADH^+$.
3. Incubate samples for 10 min at room temperature to reduce FMN. Place a vial into the luminometer, and start the enzyme reaction by automatic injection of 100 µl 0.1% n-decanal emulsion into the vial. Measure PEAK values in light units (LU) that are proportional to the amount of luciferase enzyme present in the extract.

Note: To calibrate light units to defined amounts of luciferase, homogeneous enzyme can be obtained from A.A. Szalay, Plant Molecular Genetics and Biotechnology Center, 6–30 Medical Science Building, University of Alberta, Edmonton, AB T6G 2H7, Canada. 20 LU measured by Biolumat LB9500C corresponds to 1 ng bacterial luciferase. 1 µg commercial *Vibrio harveyi* luciferase available from Sigma contains on average 1.6 ng luciferase enzyme. Advanced equipment (e.g. such as the Luminograph LB980 from Berthold Co.) not only provides a higher sensitivity, but also permits a quantitative measurement and simultaneous recording of video image of luciferase activities in living cells (Fig. 2).

By insertion of a minimal TATA-box promoter into the single *Bam* HI site separating the reporter genes from the right T-DNA border in pPCV6NFHyg, pPCVT-GUS and pPCV6NFluxF, the gene fusion vectors can be converted to enhancer tagging vectors. The use of enhancer tagging vectors is analogous to that described above (for examples of applications see [24, 25]).

Both gene fusion and enhancer tagging approaches are expected to result in recessively inherited 'loss of function' type mutations, because dominant mutations are rather infrequent in diploid plant species. Genetic studies of T-DNA insertion mutants thus require the regeneration of transgenic plants and the analysis of M2 seed progeny. Although excellent tissue culture and transformation protocols exist for some (allo)tetraploid species, such as *Nicotiana* and *Medicago*, that facilitate the application of gene fusion techniques, the use of mutant selection techniques in these systems is very difficult. However, mutations induced by T-DNA tagging in haploids are phenotypically expressed. Thus, when a suitable transformation system is available (such as in *Nicotiana plumbaginifolia*), the application of gene fusion and enhancer tagging vectors can be combined with different mutant selection techniques in tissue culture. Although haploid protoplasts tend to undergo nuclear division in the absence of cell division, and many gene mutations are expected to cause lethality, T-DNA tagging in haploids may work as efficiently as, for example, *Ty* transposon tagging in yeast.

Isolation of dominant mutations by T-DNA tagging

To overcome the difficulty of mutant selection during tissue culture transformation of plants with 2n or higher ploidy, a technology for the isolation of dominant, 'gain of function' type of mutations was developed recently. This technique is based on the application of strong enhancer elements linked to the T-DNA ends which, when integrated into the plant genome, cause *cis*-dominant activation of genes located in the vicinity of T-DNA integration sites.

1. A vector that carries 4 copies of the −90 to −440 enhancer domain of Cauliflower Mosaic Virus 35S promoter (CaMV35S) at the right T-DNA border, was thus successfully used for isolation of *Nicotiana tabacum* mutants that are capable of cell division in the absence of auxin and/or cytokinin [26]. Analogous vectors pPCVLRN4 and pPCVRN4 are shown in Fig. 1.
2. pPCVTac1 and pPCVTac7 (Fig. 1) carry a complete CaMV35S promoter with amplified enhancer domains at the T-DNA ends, and illustrate a further variation of this technology. These vectors initiate transcription of plant DNA sequences located downstream of the T-DNA border junction, as well as induce the transcription of neighboring plant genes by *cis*-dominant effect of the CaMV enhancers. These T-DNA tags may thus cause i) enhancer-mediated induction of gene expression, ii) synthesis of antisense transcripts, iii) transcriptional readthrough, and iv) transcriptional interference.
3. To obtain conditional phenotypes, thus avoiding possible deleterious effects (e.g. caused by the expression of antisense RNAs), vectors pPCVL-tx, pPCVR-tx, pPCVL-txtet and pPCVR-txtet (Fig. 1) provide two types of T-DNAs with repressable and conditionally active CaMV35S promoters. These constructs are based on a system developed by Gatz *et al.* [48] making use of the TetR repressor and TetO operator of the transposon Tn *10* tetracycline gene to achieve regulated gene expression in plants. Whereas pPCVL-tx and pPCVR-tx vectors carrying the CaMV35S-TetO promoter are useful when the target plant already contains an active TetR repressor gene, vectors pPCVL-txtet and pPCVR-txtet provide both functions in a single T-DNA for conditional regulation of gene expression by insertion mutagenesis.

Isolation of T-DNA tagged genes

Once a mutant has been identified by T-DNA tagging, a sufficient amount of material should be generated to rescue the tagged plant gene. This is no problem when M2 seed progeny is available, whereas in other cases tissue culture techniques described above can help with the amplification of calli, shoots or roots.

1. Many alternative methods are available for DNA purification [49]. We prefer to use the methods of Dellaporta [50] and Bedbrook [51] for purification of high quality total or nuclear DNA in combination with proteinase K treatment and CsCl-ethidium bromide equilibrium density gradient centrifugation.
2. The importance of careful physical and genetic mapping of T-DNA inserts cannot be overstressed. Serious drawbacks can result from the absence of precise data establishing genetic linkage between T-DNA inserts and mutations, and by the lack of appropriate physical mapping of the mutant loci. Methods for genetic analysis are described in [52], whereas techniques for physical mapping of T-DNA inserts and flanking genomic DNA by Southern DNA hybridization are listed in [37]. To obtain suitable information for plasmid rescue, cleavage sites for those restriction endonucleases that do not cut, or cut only once in the T-DNA are identified and mapped to the vicinity of inserts by the use of end-fragments of the T-DNA, as hybridization probes. For successful rescue of whole T-DNAs, or T-DNA segments carrying a pBR replicon, suitable cleavage sites for an enzyme should be located within 1 to 4 kb from the T-DNA ends.
3. Digest 5 to 20 µg plant DNA with at least a 2-fold excess of appropriate endonuclease in 100 to 200 µl volume for at least 2 h. Check whether the digestion is complete on a mini-gel using 1/10 of the digest. Purify the DNA by phenol/chloroform extraction, and precipitate by ethanol or i-propanol. Dissolve the DNA in H_2O and self-ligate at a final DNA concentration of max. 20 µg/ml [37]. Precipitate the DNA two times by i-propanol, wash by 70% ethanol, and dissolve in H_2O at a concentration of 10 to 100 µg/ml.
4. Prepare *E. coli* cells for electroporation:
   - Inoculate 1 l LB with 5 ml of an overnight *E. coli* culture, grow bacteria at 37 °C to OD_{550}: 0.5 (1×10^8 cells/ml), and harvest the cells by centrifugation at 4 °C;
   - resuspend the cells in 500 ml 1 mM HEPES (pH 7.0) at 0 °C and pellet them again, repeat this step once more;

- resuspend the cells in 10 ml 1 mM HEPES, 10% glycerol and pellet them by centrifugation. Finally resuspend the cells in 2 to 3 ml 1 mM HEPES (pH 7.0), 10% glycerol;
- mix 40 µl *E. coli* suspension and 10 µl DNA, transfer to precooled electroporation cuvettes, and electroporate the DNA in the cells as described [53]. The electroporation conditions for Biorad Gene Pulser are: 25 µF, 2.5 kV, 200 Ω, and τ 4.8 msec. Following electroporation add 1 ml SOC medium to the cells [53], incubate them for 1 h at 37 °C, and plate on LB medium containing 100 µg/ml ampicillin.

5. *Note*: Plasmid rescue is very reproducible and efficient with most standard *E. coli* hosts (e.g. MC1061, DH5α, etc.), and yields intact T-DNA plasmids rescued from *Arabidopsis*, provided the flanking plant DNA does not carry GC-rich repeats. When the T-DNA is flanked by repeats, as is frequently case in *Nicotiana*, the rescue often results in rearranged plasmids even in *mcr*, *rec*A, B, J and *sbc* mutants strains (e.g. SURE from Stratagene). In addition to inverse or RACE PCR techniques (see [44], and other chapters in this volume), an efficient method to overcome this problem is the construction of a subgenomic library by cloning of size selected plant DNA fragments in λ vectors, such as λZAP or λgt10. Using oligonucleotides that are complementary to terminal DNA sequences of λ vector arms and T-DNA ends, plant DNA sequences flanking the T-DNA can be readily amplified by PCR (i.e. polymerase chain reaction) techniques [26, 39].
6. Plant DNA fragments flanking the T-DNA are dissected from the rescued clones (or obtained by PCR amplification) and used as hybridization probes:
   - to detect RFLP (restriction fragment lenght polymorphism) between DNAs prepared from wild type, and transgenic plants heterozygous and homozygous for the T-DNA induced mutation;
   - to characterize mRNAs synthesized from the T-DNA tagged and corresponding wild type locus (for methods of RNA isolation and hybridization see other chapters of this volume and [37]); and
   - to isolate wild type cDNA and genomic clones corresponding to the T-DNA tagged locus. cDNA clones are used further as probes for mapping the genomic clones. Determination of the nucleotide sequence of both genomic and cDNAs, and mapping the 5′ end of the transcript by S1 nuclease or RNAse protection, or primer extension experiments (for methods see [37]) are usually the next step in this procedure.

Analysis of transcription and complementation of T-DNA tagged genes

Promoter and enhancer cloning vectors shown in Fig. 3 offer tools for the characterization of transcriptional regulatory elements from T-DNA tagged genes.

1. Full-length promoters, or their 5′ to 3′ *cis*-deletion derivatives, e.g. obtained by exonuclease III generated nested deletions used for DNA sequencing [37], can be dissected from the rescued T-DNA reporter gene fusions and cloned into different sites of polylinkers located upstream of *aph*(3′) II, *uid*A and *lux*F reporter genes in the promoter cloning vectors pPCV811, 812, and 813.
2. Alternatively, 5′-upstream regulatory sequences of plant promoters can be fused to minimal TATA box promoters derived e.g. from either the *iaa*H gene of the T_L-DNA [54], or the promoter of CaMV 35S RNA [23, 55], in the enhancer test vectors pGDW44, pGDW4421 and pETV-gus. The activity of reporter genes driven by such recombinant promoter constructs can be studied in transgenic plants as described for the gene fusion vectors.

A common goal of transformation experiments is to express sense or antisense transcripts, if possible in a regulated fashion, in transgenic plants. Expression of wild type genomic or cDNA clones in T-DNA insertion mutants thus aims at a phenotypic complementation of T-DNA induced mutations, whereas expression of antisense transcripts in wild type plants is expected to result in the appearance of mutant phenocopies.

3. Prototypes of expression vectors depicted in Fig. 4 offer gene cassettes with various plant promoters and polyadenylation signals that can efficiently be used for gene expression and mutant complementation studies, as described [20, 23, 46, 56].

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