Chapter 3
T-DNA tagging

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1 Introduction

This Chapter gives a guide to the use of *Agrobacterium* T-DNA as an insertion mutagen in the molecular genetic analysis of plant gene functions. T-DNA represents a segment of Ti and Ri plasmids that is transferred from *Agrobacterium* into the nuclei of infected plant cells where it is randomly integrated into potentially transcribed domains of the chromosomes by non-homologous (i.e. illegitimate) recombination (1, 2). Insertion of T-DNA into plant genes causes gene mutations. Hence, T-DNA is an efficient mutagen (3), see also Chapter 1. T-DNA tagging, which results in the labelling of gene mutations with known sequences and dominant selectable markers, provides a simple means for genetic linkage mapping, functional analysis and molecular characterization of plant gene mutations (4, 5).

Promoterless reporter genes, with or without a translation initiation codon, are inserted into plant genes with the aid of the T-DNA. Thus, gene mutations are identified by selection or screening for the expression of transcriptional or translational reporter gene fusions controlled by T-DNA tagged plant genes (5). Reporter genes bearing only the TATA-region of so-called minimal promoters are used as enhancer traps to identify *cis*-acting regulatory sequences with the help of T-DNA tags in the plant genome (6).

Mutagenesis with T-DNAs, carrying multiple copies of enhancers derived, for example, from the well-known promoter of the Cauliflower Mosaic Virus (CaMV) 35S RNA, are employed to induce *cis*-activation of plant genes located in the vicinity of T-DNA tags (7). This activation T-DNA tagging technology is used for the isolation of dominant mutations that can also be identified in tetraploid and allotetraploid plants. Promoters orientated towards the termini of T-DNA tags provide a means for induction of either constitutive or regulated transcription of neighbouring plant DNA sequences. This may either activate or inactivate genes by transcriptional read-through or interference, as well as lead to alterations in chromatin structure implying long-range *cis*-effects (8).

Applications, aiming at either a removal of certain T-DNA tags from the genome or isolation of a particular type of gene mutation apply conditional

'suicide' genes for negative selection (9–11). A combination of these methods facilitates the isolation of regulatory mutations that affect the activity of single genes, including second site suppressors of known mutations. T-DNA insertions carrying target sites and genes for site-specific recombination also allow chromosome engineering by generation of defined deletions, additions, inversions and translocations (12, 13). Finally, T-DNA serves as a common vehicle to deliver autonomous or defective, but mobilizable, transposons and retrotransposons into diverse plant species as described in Chapter 4.

2 Generation of T-DNA transformants

2.1 The mechanism of T-DNA transformation

The virulence of *Agrobacterium* strains is regulated by the bacterial *vir* loci of Ti and Ri plasmids, and by a few chromosomal genes. The VirA chemosensor histidine protein kinase controls the activation of the VirG transcription factor that binds to *vir*-boxes in the promoters of virulence genes as a positive effector. Activation of the VirA kinase is induced by plant phenolic compounds and sugars, and shows a considerable variation between different *Agrobacterium* isolates. VirG activation by VirA-mediated phosphorylation induces the synthesis of Vir proteins required for T-DNA transfer into plant cells.

The mechanism of T-DNA transfer is analogous to the conjugation of bacterial plasmids, except that it works with two conjugation transfer origins (ori_T) defined by T-DNA 25 bp border repeats (1). During T-DNA transfer, a single-stranded DNA intermediate (T-strand) is produced by strand-replacement DNA synthesis, which is initiated by a single-strand nick at the right T-DNA border and terminated by a similar nick at the left 25 bp border sequence. The VirD2 pilot protein covalently binds to the 5′-end of the T-strand, which is packaged in its entire length by the VirE2 single-stranded DNA-binding protein and transferred into plant cells through pores formed by the VirB proteins. Nuclear localization signals present in VirD2 and VirE2 aid the import of the T-complex into plant cell nuclei (1). Although the plant factors involved in the T-DNA integration are still unknown, the available data show that T-DNA integration requires the function of the VirD2 nicking-closing enzyme and occurs by illegitimate recombination targeting either single-stranded nicks or double-stranded breaks (2). The T-DNA integration process usually generates small target site deletions (extending from one to several hundred base-pairs), but may also cause larger rearrangements such as translocations and inversions. Because the T-DNA integration process is based on DNA replication and repair, it is common that aborted integration events generate footprints causing mutations that are not genetically linked to T-DNA tags.

2.2 *Agrobacterium* hosts

Infection with *Agrobacterium* strains carrying wild type Ti or Ri plasmids leads, respectively, to neoplastic tumour (crown-gall) formation or hairy-root syn-

drome in many dicotyledonous plants. These disease symptoms result from the expression of T-DNA encoded genes that play no role in the T-DNA transfer process, but cause alterations in the regulation of the hormonal, chiefly auxin and cytokinin, balance of transformed plant cells. Initially, the absence of these typical disease symptoms suggested that monocotyledonous plant species are not suitable hosts for *Agrobacterium*. This view was indeed supported by the identification of several compounds in monocots that inhibit the induction of *Agrobacterium vir* genes. To increase the efficiency of the T-DNA transfer process, if possible independently of the host signals, several wide-host range Ti plasmids were studied. New *Agrobacterium* strains were constructed by engineering of the VirA kinase and over-expression of the VirG regulator that, when produced in excess, can mediate a VirA-independent activation of the virulence genes. To monitor the T-DNA transformation process, intron-containing reporter genes (e.g. *uidA*) silent in *Agrobacterium* were developed (for review see 14), see also Chapter 2.

A break-through resulting from these efforts demonstrated that (i) *Agrobacterium* can transform virtually all plant species, including the most important monocotyledonous crops, and (ii) *Agrobacterium* is capable of systemically infecting most plant tissues, including the reproductive plant organs, protoplasts, cell suspensions, somatic embryos, embryogenic organ cultures, etc. In addition to a huge number of dicots providing food, raw material and pharmaceutical drugs, tissue culture-based T-DNA transformation methods are now available, e.g. for rice (15), maize (16), barley (17), wheat (18), and sugarcane (19) (see Chapter 2). Furthermore, T-DNA transfer into yeast, filamentous fungi and mammalian cell nuclei has been recently achieved thus widely extending the applicability of T-DNA-based gene transfer and insertion mutagenesis technologies (20–22).

3 T-DNA tagging in *Arabidopsis*

To give a guide to the application of T-DNA insertion mutagenesis in plant molecular biology, we describe here the use of T-DNA transformation methods in the model plant *Arabidopsis thaliana*. These methods are adaptable to any plant species for which a suitable *Agrobacterium*-mediated transformation protocol is available yielding fertile transgenic plants.

3.1 Generation of transformants

3.1.1 Transformation of explants

In *Arabidopsis*, as in all other species, the use of tissue culture transformation techniques was first elaborated. These techniques require an initial amplification of *Agrobacterium* transformed cells. This can be conveniently achieved by selection for transformed micro-calli that can then be regenerated into shoots and plants. Therefore, the choice of explants for co-cultivation with *Agrobacterium* largely depends on the response of diverse plant tissues to those

hormonal treatments that are employed to induce cell proliferation and subsequent shoot formation. In general, cell proliferation is induced by culturing the explants in media providing a high auxin to low cytokinin ratio. Explants from various *Arabidopsis* ecotypes and mutants respond differently to auxin and cytokinin, and therefore to the induction of cell division. Thus, roots of the ecotype Columbia (Col-0) proliferate much faster than tissues from hypocotyl, cotyledon, petiole, or leaf, whereas acceptable callus formation is achieved from most explants of ecotypes RLD and C24. In comparison, ecotype Landsberg *erecta* (Ler-0) shows a very poor tissue culture response (8). Throughout the last decade, several methods for root transformation were published (14). *Protocol 1* describes a method that has been used by our laboratory to generate a large number of transgenic *Arabidopsis* plants and to search for recessive gene mutations using the T-DNA-aided gene fusion technology.

Protocol 1

Agrobacterium-mediated transformation of *Arabidopsis* root explants

Equipment and Reagents

- Macro-salts: 20 g NH_4NO_3, 40 g KNO_3, 7.4 g $Mg_2SO_4.7H_2O$, 3.4 g KH_2PO_4, and 2 g $Ca(H_2PO_4)_2$ for 1 l
- Micro-salts: 6.2 g H_3BO_3, 16.9 g $MnSO_4.H_2O$, 8.6 g $ZnSO_4.7H_2O$, 0.25 g $Na_2MoO_4.2H_2O$, 0.025 g $CuSO_4.5H_2O$, 0.025 g $CoCl_2.6H_2O$ for 1 l
- Fe-EDTA: dissolve separately 5.56 g $FeSO_4.7H_2O$ and 7.46 g $Na_2EDTA.2H_2O$; mix the two solutions, and adjust the volume to 1 l
- $CaCl_2$: 7.5 g $CaCl_2.2H_2O$ per 1 l.
- KI: 375 mg KI per 1 l.
- Vitamins: 50 g *myo*-inositol, 2.5 g thiamine.HCl, 0.5 g nicotinic acid, and 0.2 g pyridoxine.HCl for 1 l.
- MSAR-medium: to prepare liquid medium, add for 1 l: 50 ml macro-salts, 1 ml micro-salts, 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 with 0.5 M KOH. To prepare solid MSAR media, add for 1 l, either 2.2 g phytagel (essential for high-frequency *Arabidopsis* shoot regeneration; Sigma) or 8 g high quality agar for plant tissue culture. Autoclave the media for 15 min.
- Seed germination (SG) medium: regular MSAR medium, but contains half concentration of macro-salts and 0.5% sucrose.
- Callus medium (MSARI): MSAR medium supplemented with 2.0 mg/l indole-3-acetic acid (IAA), 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg/l kinetin (6-furfurylaminopurine), and 0.2 mg/l N^6-[2-isopentenyl]-adenosine (IPAR).
- Shoot medium (MSARII): MSAR medium supplemented with 2.0 mg/l IPAR and 0.05 mg/l 1-naphtaleneacetic acid (NAA).
- Rooting medium (MSARIII): MSAR medium supplemented with 0.2 mg/l 6-benzylaminopurine (BAP), 0.1 mg/l indole-3-butyric acid (IBA) and 0.05 mg/l NAA.

Protocol 1 continued

- Seed sterilization solution: 5% $Ca(OH)_2$ containing 0.05% Triton X-100. If $Ca(OH)_2$ is not available, use 10% sodium hypochlorite solution containing 0.1% Triton X-100.
- *Agrobacterium* YEB-medium: 5 g beef extract, 1 g yeast extract, 5 g peptone (each from Difco Laboratories) and 5 g sucrose per 1 l water

Methods

A. Preparation of plant material

1 Sterilize 10 mg (about 500) seeds in a microfuge tube after adding 1 ml seed sterilization solution by continuous shaking for 15 min.

2 Pellet the seeds by brief centrifugation (i.e. 1 min in a microfuge at room temperature), remove the sterilization solution, wash the seeds with 4 × 1 ml sterile water, and dry the tubes in a flow-hood overnight.

3 Germinate the seeds on solid SG-medium in Petri dishes for 7–10 days using an 8 h light and 16 h dark period at 25 °C.

4 Place 20 seedlings into a 250 ml Erlenmeyer flask containing 35 ml liquid MSAR-medium with 3.0% sucrose and fix the flasks onto a rotary shaker adjusted to 120 r.p.m. at 25 °C under an 8 h light and 16 h dark period. Within 14–20 days the flasks will be filled with roots. This material can be used either for direct infection with *Agrobacterium* or for generation of cell suspensions as described in *Protocol 2*. Do not collect roots from plants that show senescence.

B. Growth of Agrobacterium

1 Grow *Agrobacterium* to an OD_{550} of 1.5 in YEB-medium supplemented with proper antibiotics to select for the maintenance of binary T-DNA vectors.

2 Pellet the cells by centrifugation (5000 *g* for 15 min at room temperature).

3 Resuspend cells in MSARI liquid medium at an OD_{550} of 0.5 and distribute 50–80 ml aliquots into large Petri dishes.

4 Collect *Arabidopsis* plants from the Erlenmeyer flasks (see *Protocol 1, A4*), remove their roots, cut the roots in small pieces and place them into the *Agrobacterium* suspension for at least 30 min.

5 Remove the *Agrobacterium* suspension with a pipette from the Petri dishes and plate the roots onto MSARI plates.

6 Alternatively, collect the root segments in an Erlenmeyer flask containing 40 ml MSARI medium and place the flask on a rotary shaker set as described above. Co-culture the roots with *Agrobacterium* for 36–48 h.

Protocol 1 continued

C. Selection of transformed plants

1 Transfer the roots from MSARI to MSARI-medium containing 300 mg/l claforan (cefotaxime) and 500 mg/l tricarcillin/clavulanic acid (15:1; Duchefa Biochemie). Alternatively, add claforan alone at 500mg/l concentration.

2 Subculture the liquid root cultures every 3–4 days using the medium above.

3 Root cultures in Petri-dishes do not need to be subcultured. After 14 days, transfer the roots onto MSARII plates containing claforan (300 mg/l), tricarcillin/clavulanic acid (500 mg/l) and, depending on the type of selectable marker encoded by the T-DNA, for example, 15 mg/l hygromycin (Boehringer Mannheim) or 100 mg/l kanamycin (Sigma).

4 Subculture the root explants at 14 days intervals by decreasing the claforan concentration to 300 mg/l after the third subculture.

5 During the first 4 weeks on selective MSARII medium the non-transformed tissues die and green transgenic calli appear on the plates. Within 8 weeks after *Agrobacterium* infection, the transformed calli form embryo-like structures and quickly regenerate to shoots.

6 The regenerating shoots must be continuously transferred onto MSARIII medium in jars and, when they reach a size of 1–2 cm, into large test tubes (e.g. 20 × 2.5 cm diameter) containing 10–20 ml SG-medium to set seed.

7 The test tubes are closed with loose cotton plugs and placed in growth chambers providing a 16 h light and 8 h dark cycle.

8 Typically, root culture from a single Erlenmeyer flask yields explants for 10–12 MSARII plates resulting in an average of 500 to 1000 transformed plants.

3.1.2 Transformation of cell cultures

An advanced version of tissue culture transformation methods is based on the co-cultivation of cell suspensions with *Agrobacterium* that yields an extremely high number of transformants (in the range of 10^6–10^7). Therefore, this method ideally suits the purpose of activation T-DNA tagging, if a proper selection for gene activation is available, for example, using inhibitors of particular enzymes or signalling factors, and plant regeneration is not immediately essential. Although embryogenic cell suspensions can be established with care and transformed similarly by *Agrobacterium* as described in *Protocol 2*, the regeneration of large numbers of transformants is a very laborious process. Moreover, alterations in ploidy (from 2n to 4n or 8n) are unavoidable in cell cultures maintained in the presence of high concentrations of auxin (i.e. 2,4-D). An increase in the ploidy level reduces the recovery of recessive mutations (see also Chapter 1), but permits the identification of transformants that carry dominant mutations induced by activation T-DNA tagging.

Protocol 2

Establishment and transformation of Arabidopsis cell suspensions

Equipment and Reagents

- SC-medium: 4.33 g MS-medium (Sigma), a double concentration of B5 vitamins (Sigma, 2 mg nicotinic acid, 2 mg pyridoxine-HCl, 20 mg thiamine-HCl, and 200 mg *myo*-inositol for 1 l), 30 g sucrose, 0.5 mg/l 2,4-D, 2 mg/l IAA, and 0.5 mg/l IPAR. The vitamins and hormones are prepared as concentrated stocks, filter sterilized, and added to SC after autoclaving.
- Metal sieve: pore size 250 and 850 μm, available from, e.g. Fastnacht Laborbedarf GmbH, Bonn, Germany

Methods

A. Initiation of Arabidopsis cell suspension

1 Continue *Protocol 1A* from step 4. Transfer the roots from an Erlenmeyer flask into a Petri-dish, remove all green tissues.

2 Cut the roots into about 2-mm pieces, and transfer the explants into a 250 ml Erlenmeyer flask that contains 50 ml suspension culture SC-medium. Cover the flasks with aluminium foil and place them on a rotary shaker (120 r.p.m., 24 °C).

3 After 15–20 days, approximately 3–5 g roots (fresh weight) should be available in each flask.

4 Exponential growth rate of roots is important for obtaining fine cell suspensions. Therefore, avoid yellow-brown stationary phase cultures.

B. Subculture of cell suspension

1 After 21 days, harvest the micro-calli released from the roots by passing the root culture medium through a 850 μm sterile metal sieve into a sterile Erlenmeyer flask.

2 Pellet the cells, and replace half volume of the medium with new SC-medium.

3 Add 50 ml fresh SC-medium to the roots and culture them further for 21 days to generate more micro-calli.

4 Subculture the cell suspension every 7 days by sieving and replacing half of the medium with new SC-medium after pelleting the cells.

5 After the fifth subculture, a 50 ml suspension can usually be diluted with 100 ml SC and divided into three equal aliquots into new flasks for amplification.

C. Transformation of cell suspension with Agrobacterium

1 Logarithmically proliferating cell suspensions are usually obtained from *Arabidopsis* ecotype Col-0 after 5–6 weeks.

2 The cell suspensions are then filtered through a 250 μm sieve and subcultured twice.

Protocol 2 continued

3 The resulting fine cell suspension is inoculated with *Agrobacterium*. For this purpose, grow *Agrobacterium* in YEB-medium as described in *Protocol 1B* and pellet the cells from 1.5 ml culture in a sterile microfuge tube by centrifugation for 2 min at room temperature.

4 Suspend the bacterial pellet in 1.5 ml SC-medium and add to 35 ml cell suspension immediately after subculturing.

5 Co-cultivate the cells with *Agrobacterium* for 48 h, then add claforan (300 mg/l) and tricarcillin/clavulanic acid (300 mg/l).

6 Subculture the cells 7 days after the addition of *Agrobacterium* in the same SC-medium.

7 After 1 week transfer the cells into a 50 ml Falcon tube, pellet the cells by centrifugation at 1000 *g* for 3 min and suspend them at a density of 10^3 cells/ml in either 0.2% phytagel dissolved in 3% sucrose or in liquid MSARII medium.

8 Plate the cells on MSARII medium containing 0.2% phytagel, claforan (200 mg/l), tricarcillin/clavulanic acid (200 mg/l), and suitable antibiotics (e.g. hygromycin or kanamycin) as described in *Protocol 1*.

9 If you wish to select for transformed calli without plant regeneration, use MSARI instead of MSARII medium for plating.

10 Transformed calli appear within 2-3 weeks and yield shoots within 35-40 days following the *Agrobacterium* infection.

When using this protocol for selection of mutants resistant to various inhibitors, a proper adjustment of selection conditions should first be performed to ensure 100% killing of non-transformed cells. Some inhibitors are light sensitive, thus they can only be used in combination with MSARI medium permitting the selection of transformed calli in the dark. Further details to this protocol are described by Mathur *et al.* (23).

3.1.3 *In planta* transformation

The first successful approach using a large-scale *in situ* infection of *Arabidopsis* with *Agrobacterium* was the seed transformation method (24). With this method, a larger population of transformants could be raised with less labour than using tissue culture techniques. Furthermore, seed transformation overcame the problems of recognition and removal of polyploids and aneuploids during the genetic analysis (25). The recognition that *Arabidopsis* survives systemic *Agrobacterium* infection led to the development of *in planta* transformation techniques using vacuum infiltration (26). Because the transformation process must target the reproductive organs, this technology was further perfected by dipping only the flower buds into *Agrobacterium* suspension containing 5% sucrose and Silwet L-77 as surfactant (27).

To amplify the number of inflorescence axes produced by a plant for *Agrobacterium* infection, the primary inflorescence stem is often removed. This

results in a quick outgrowth of side-inflorescence stems from the rosette providing ample material for transformation. Most binary vectors used currently are devoid of proper partitioning functions, which would provide stable plasmid maintenance. Therefore, to increase the transformation frequency, an application of *in planta* selection for the maintenance of *Agrobacterium* binary T-DNA vectors is advisable. In the case of the pPCV vectors system developed in our laboratory (8), the T-DNA carries an ampicillin/carbenicillin resistance gene. The media for *Agrobacterium* infiltration and floral dip transformation can therefore be supplemented with carbenicillin, which is not deleterious to *Arabidopsis*.

By optimization of the infiltration techniques, on average over 400 transformed seeds are routinely obtained from a single pot containing 10–15 plants. Using 500–1000 pots, one can reproducibly raise a T-DNA mutagenized population of 200 000 transformants within 3–6 months. Based on the expectation that T-DNA integration occurs randomly and the size of *Arabidopsis* genome is 120 Mb, such a population is predicted to carry a T-DNA insert at about every 0.5 to 1 kb in the genome. Therefore, T-DNA tagging may be used as an alternative to chemical mutagens (see Chapter 1) to solve specific genetic problems, such as the isolation of multiple alleles and suppressors for a given mutation.

According to the type of selectable marker gene within the T-DNA, different techniques are used for the selection of primary transformants in the M1 generation. The application of the BASTA resistance marker facilitates the selection of transformed plants in growth chambers by spraying with the herbicide. In contrast, hygromycin or kanamycin selection can be optimally carried out by germination of seeds in large Petri-dishes which, although somewhat more costly, saves considerable time and greenhouse space for a more economic production of the M2 seed generation. Suitable amounts of plant material for subsequent sequence-based PCR screens (see *Protocol 4*) may be collected immediately if the M1 generation is grown initially in short day conditions, which promotes large rosettes. In practice, growing of single plants in separate quadratic pots (e.g. 5 × 5 cm) yields as much as 0.5–1 g of leaf material that can be pooled for DNA preparations, as well as a large amount of seed essential for further genetic analyses.

Protocol 3

In planta transformation of *Arabidopsis* by infiltration

Equipment and Reagents

- Agrobacterium YEB-medium: see *Protocol 1*
- SG-medium: see *Protocol 1*
- Infiltration medium (IM, pH 5.7): half a concentration of Murashige and Skoog basal salt mixture (Sigma), 1 × B5 vitamins (Sigma), 5% sucrose, and is supplemented after autoclaving with 0.05 μM BAP, and 0.005% (volume/volume) Silwet L-77 (Ambersil)

Protocol 3 continued

Methods

A. Growth of plant material

1 Prepare plastic pots (12 cm diameter) with wet soil and sow 10–15 seeds in each.

2 Grow seedlings in short days using an 8 h light (24 °C) and 16 h (18–20 °C) dark period in a growth chamber or greenhouse.

3 When the diameter of rosettes reaches a size of 3–5 cm (usually after 15–18 days), transfer the pots to long day conditions (16 h light and 8 h dark) to induce flowering.

4 Cut off the primary inflorescence stem when it reaches 1–3 cm, to induce the development of secondary inflorescence from the rosette buds.

5 When the size of inflorescence reaches 5–10 cm, carrying closed flower buds and only a few open flowers, the plant material is ready for transformation.

B. Growth of Agrobacterium

1 Grow 100 ml liquid culture of *Agrobacterium* in YEB medium at 28 °C for 2 days applying a proper selection for the maintenance of binary T-DNA vectors.

2 Best transformation results were reported by using the strain GV3101 with the helper Ti-plasmid pMP90 (26–28). A modified version of this *Agrobacterium* host, GV3101 (pMP90RK) is used in combination with the pPCV-type binary T-DNA vectors that carry a carbenicillin resistance gene within their T-DNA (28).

3 When using pPCV-vectors, supplement both *Agrobacterium* and *in planta* infiltration media with 100 mg/l carbenicillin.

4 Inoculate with 10 ml starter culture several 2-l Erlenmeyer flasks containing 500 ml YEB-medium and grow the bacterial cultures to late logarithmic phase for 12–14 h. Harvest the cells by centrifugation (5000 g for 10 min at room temperature) and resuspend the cells at an OD_{550} of 0.8–1.2 in infiltration medium. Typically, 500 ml *Agrobacterium* culture yields 1 l bacterial suspension for infiltration.

C. Vacuum infiltration

1 Assemble a glass or plastic bell jar with a vacuum pump and place trays (for example, 30 × 10 × 5 cm), or beakers (12 cm) containing the *Agrobacterium* culture (*Protocol 3B*) into the jar.

2 Place two pots in inverted position into each tray, or one pot inverted into each beaker such that only the inflorescence shoots are submerged into the *Agrobacterium* suspension. Apply vacuum (e.g. 16 mbar) for 5 min, then release the vacuum quickly by disconnecting the fitting between the pump and the bell jar.

3 The bacterial suspension can be reused 3 or 4 times. Thus, an infiltration of 16 pots needs about 1–1.5 l *Agrobacterium* suspension in IM.

4 Cover the pots for 24 h with plastic quick-seal bags with the corners cut off. If the humidity in your greenhouse is high enough (i.e. 70–80%), this step is not necessary.

Protocol 3 continued

D. Collection of seeds

1 The vacuum infiltrated plants complete flowering within 10–12 days.

2 When the last flowers are fertilized, place the inflorescence stems of five to eight plants into a translucent paper bag (i.e. 13 × 30 cm).

3 Fix the closed bags with tape to wood or plastic sticks fitted into the pots.

4 Water the plants for an additional 10 days and then let the pots dry out.

5 Collect the seed-bags and clean the seeds.

6 Take 5–10 seed aliquots derived from different pots to test the transformation efficiency in a germination assay.

E. Seed sterilization

1 Sterilize seeds as described in *Protocol 1A* and sow them on SG-medium in Petri-dishes (15 cm diameter) containing suitable antibiotics to select for T-DNA transformed plants.

2 Using hygromycin selection (25 mg/l, Boehringer), non-transformed seedlings are arrested and show bleaching after opening their cotyledons, whereas transformed plants develop leaves and long roots within 5–8 days.

F. Selection of transformants

1 If at least 5–10 transformants were obtained from 500 to 1500 seeds, bulk the seed material, sterilize it in aliquots, and plate the seeds at a high density on selective SG-medium with 1000–5000 seeds per large Petri-dish.

2 To save greenhouse space, the transformants picked from Petri-dishes can be grown in jars on SG-medium with hygromycin, and when required with claforan to eliminate possible *Agrobacterium* infection, under short day conditions for another 10 to 14 days.

3 Transfer the seedlings into soil by planting them individually into 5 × 5 cm (or 7 × 7cm) quadratic pots and grow them for 10–14 days under short day conditions to produce large rosettes (average diameter 6–8 cm).

4 Collect rosette leaves (200–500 mg) for DNA purification using a pooling strategy (4), and transfer the plants to long day conditions to collect M2 progeny from each plant separately within 3–4 weeks.

4 Screening approaches and theoretical considerations

4.1 Phenotype-based screening

Mathematical theories of mutagenesis experiments and classical mutant screening strategies are extensively reviewed in recent *Arabidopsis* handbooks (29, 30;

see also Chapter 1). Dominant mutations affecting, for example, the development of organs, epidermal hair shape and distribution, wax deposition, colour, male and female fertility, and seed morphology are rare in T-DNA tagged populations. Nonetheless, the M1 population should be inspected to score for potential mutants. Once such a potential dominant mutant is found, it should immediately be out-crossed with wild type to speed up the genetic analysis. The application of gene fusion and enhancer trap technologies provides the unique advantage that the M1 population can be screened directly for T-DNA tags in genes or in the vicinity of regulatory DNA sequences, respectively. M1 seedlings and various plant organs can thus be used for monitoring the activation of reporter genes encoding β-glucuronidase (GUS), firefly luciferase (luc) or green fluorescent protein (GFP) *in vivo* and *in vitro* (see Volume 2, Chapter 13).

From a genetic point of view, the examination of immature seeds representing the M2 population in fruit capsules (i.e. siliques) is the most meaningful screening approach in the M1 generation. Using classical 'm' tests (31), at least two siliques are opened and examined for Mendelian segregation of the progeny to find defects in embryo development, embryo lethality, albino, or purple (i.e. *fusca*) colour, abnormal shape and size, viviparity, or alteration in starch, protein, fat, metabolite, hormone levels, etc. In addition, pollen may be collected to perform morphological examinations and *in vitro* germination assays.

Most mutant screens are carried out by germinating M2 seeds on diverse media in either dark or light. It must be noted that *Arabidopsis* seeds require a red light stimulus to germinate synchronously and need vernalization, depending on the ecotype. Dark germination assays combined with the exposure of seedlings to different light sources is used to screen for mutations affecting growth responses to red, far-red, blue, and UV light, or a timely combination of these. Hypocotyl and root elongation, opening the cotyledons, growth responses to gravitropic stimuli, hormones, hormone antagonists, stress factors, sugars, nitrate, ammonia, salts, osmolytes, volatile substances, and many other compounds can be assayed simply in both dark and light germination tests at different temperature regimes. Defects of seed germination, alterations in morphology, colour, elongation, differentiation and development of diverse organs, changes in cell size, number and pattern in various tissues, for example, shoot and root meristems, are also scored in simple germination assays. By modification of the growth medium, the seedlings can also be exposed to various combinations and concentrations of inducers and inhibitors of metabolic and signalling functions, suicide enzyme substrates, cytotoxic and genotoxic agents, or labelled compounds in uptake assays providing a wide choice of phenotypic screens. These screening strategies are illustrated by the steadily growing literature of T-DNA tagged mutants (for references see 4).

Recently, many novel cytological, biochemical and physiological screens were developed to isolate T-DNA tagged genes controlling female or male meiosis (32–36), temporal development of embryos, trichomes, and root hairs (37–41), cellular interactions with nematodes (42, 43), responses to genotoxic stress (44), cytoskeleton organization (45), membrane transport (46), and remarkably T-DNA

integration in *Arabidopsis* (47). Based on the availability of sequenced cDNAs as expressed sequence tags (ESTs) and recent progress in genome sequencing, smart phenotypic screens are gradually being replaced by systematic sequence-based screening approaches of reverse genetics.

4.2 Reverse genetics using sequence-based PCR screening approaches

Saturation mutagenesis of the *Arabidopsis* genome can be reproducibly performed using the *in planta* transformation methods. A population of 250 000 transgenic plants is probably sufficient to find a T-DNA insertion at any 0.5 kb region of the *Arabidopsis* genome (about 120 Mb), assuming that T-DNA integration is random. If an average of 1–3 T-DNA inserts is present in each transformed plant and given that the size of an average *Arabidopsis* gene is 2–4 kb, a population of 60 000 plants should already contain more than 90% of all gene mutations. Forward genetic screens, proceeding from mutant isolation to gene cloning, are thus probably less efficient than reverse genetics that uses a target-selected mutant isolation approach to establish a correlation between sequence information and mutant phenotypes (see also Chapter 4 on transposon tagging). Epistasis studies with targeted mutations also provide an excellent means for genetic confirmation of plant protein interactions identified in the yeast two hybrid system. RNA-derived probes prepared from any mutant identified by reverse genetics may also be used to probe cDNA-micro-arrays or gene-chips (see Volume 2, Chapter 3), ideally carrying DNA sequences of all *Arabidopsis* genes, in order to generate digitized molecular phenotypes and identify regulatory functions.

For systematic identification of T-DNA tagged gene mutations, PCR-based screening methods use two PCR primers oriented 5′ to 3′ toward the termini of T-DNA inserts. These T-DNA primers are combined with target-specific primers that are oriented toward a specific gene sequence from 5′ and 3′ directions. Thus, when an insertion is localized in a target gene, a PCR product is obtained with either the 5′ or 3′ gene specific primer, or both. This PCR-based mutant screening technique was first developed for the transposon Tc1 in *Caenorhabditis* and the P-element in *Drosophila* (48–51), and then successfully adapted to the identification of transposon and T-DNA tags in *Petunia* and *Arabidopsis* genes (52–57; see also Chapter 4).

The PCR screening methods are helped by various pooling strategies (see also Chapter 4). The samples, representing either single individuals, or pools of mutants derived from 10 to 100 individuals are arranged in an array to form a 10 × 10 matrix. Samples from each horizontal row and column of an array are pooled for one PCR reaction. Piles are built from the arrays to find a positive sample in a three dimensional matrix. This is usually not required for PCR-screening of T-DNA-tagged *Arabidopsis* lines. It is often sufficient to collect 200 mg leaf material from each M1 plant in order to build a 10 × 10 array in which each sample contains pooled DNA from 100 M1 plants. Thus, pooling rows and columns results in 20 PCR reactions for the screening of 10 000 plants. To find a

mutant, 100 plants consisting of a positive sample must be newly grown from M2 seed, arranged in a 10 × 10 array, and pooled in rows and columns to run 20 additional DNA preparations and PCR reactions. Thus, when a population of 100 000 plants is examined, a mutant is found by performing 10 × 20 + 20 = 220 PCR reactions. Because each sample contains 20 g of leaf material from 100 M1 plants, when DNA of high quality is prepared, the material is sufficient for the screening of several thousand gene mutations.

4.3 Identification of gene mutations using sequenced T-DNA tags

Direct sequencing of T-DNA insert junctions is a cumulative approach to sequence-based identification of T-DNA tagged genes. Methods using inverse PCR (iPCR) employ two T-DNA-specific primers that allow the amplification and subsequent bi-directional sequencing of plant DNA fragments that are linked to either the left or right T-DNA ends, or both (23). Alternative techniques, such as the asymmetric interlaced PCR or amplification of insertion mutagenized sites (AIMS), use either a degenerated primer or an adaptor in combination with T-DNA end-primers to amplify plant DNA sequences flanking the T-DNA tags (58, 59). *Protocol 4* describes the application of a long-range iPCR technique that allows routine amplification and sequencing of T-DNA-linked plant DNA fragments ranging in size from 300 bp to 12 kb.

Protocol 4

PCR amplification of T-DNA tagged plant DNA fragments for automatic DNA sequencing

Equipment and Reagents

- SG-medium: see *Protocol 1*.
- DNA extraction buffer: 200 mM Tris–HCl (pH 8.5), 50 mM EDTA, 500 mM NaCl, 14 mM 2-mercaptoethanol and 1% SDS
- HTE-buffer: 100 mM Tris–HCl (pH 8.0), 50 mM EDTA
- LTE-buffer: 10 mM Tris–HCl (pH 7.5), 1 mM EDTA
- RNase A: from SERVA, prepare 10 mg/ml stock solution in LTE-buffer and boil it for 10 min. Store at 4 °C
- Proteinase K: Merck, prepare freshly 2 mg/ml stock solution in LTE-buffer and incubate it at 37 °C for 10 min
- T4 DNA ligase buffer: 66 mM Tris–HCl (pH 7.2), 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM ATP
- Nested primers: for binary *Agrobacterium* vectors pPCV6NFHyg and pPCV621 (23) the following left (LB) and right (RB) T-DNA border primers are used:

  LB1 (5′-CTCAGACCAATCTGAAGATGAAAT GGGTATCTGGG-3′)

  LB2 (5′-CTGGGAATGGCGAAATCAAGGCAT CGATCGTGAAG-3′)

  RB1 (5′-GTTTCGCTTGGTGGTCGAATGGGC AGGTAGCC-3′)

  RB2 (5′-CAGTCATAGCCGAATAGCCTCTCC ACCC-3′)

Protocol 4 continued

- Elongase PCR buffer: 60 mM Tris-SO_4 (pH 9.1), 18 mM $(NH_4)SO_4$, 1 mM $MgSO_4$, and 250 μM of each dATP, dCTP, dTTP, and dGTP
- ABI Prism-Dye Terminator Cycle Sequencing kit: Perkin-Elmer Ltd

Methods

A. DNA preparation

1 Collect two to four leaves (50–200 mg) from M1 plants in microfuge tubes or M2 seedlings germinated on selective SG-medium.

2 Snap-freeze the tubes in liquid N_2, and grind the material to a fine powder with a glass or Teflon rod fitting the shape of the microfuge tube.

3 Add 750 μl freshly made DNA extraction buffer, mix the extracts well, and incubate the tubes with occasional shaking at 65 °C for 10 min.

4 Add 250 μl 5 M potassium acetate (pH 6.0), mix the tubes, and place them on ice for 20 min.

5 Spin the tubes in a microfuge for 3 min and in the meantime prepare 1 ml pipette tips plugged with small pieces of Miracloth (Calbiochem).

6 Filter the cleared lysates through the Miracloth-plugged tips into new tubes and precipitate the DNA with 700 μl isopropanol on ice for 30 min.

7 Collect the DNA by centrifugation in microfuge for 5 min, wash the pellet with 70% ethanol, and dissolve the DNA in 475 μl HTE-buffer containing 200 μg/ml heat-treated RNase A. Incubate the samples for at least 30 min at 37 °C.

8 Add 125 μl proteinase K solution and incubate the samples for 1 h at 37 °C.

9 Extract the samples twice with 600 μl phenol/chloroform/ isoamylalcohol (25:24:1) and with chloroform/isoamylalcohol (24:1) at 0 °C.

10 Add 60 μl 3 M sodium acetate (pH 6.0) and 400 μl cold isopropanol, mix the tubes and precipitate the DNA on ice for 30 min.

11 Pellet the DNA by centrifugation in a microfuge for 5 min and dissolve in 100 μl LTE-buffer.

This modified version of a plant DNA mini-preparation protocol from Dellaporta *et al.* (60) yields a high quality DNA with an average length of >30 kb. Some other protocols (52, 54, 56) yield less clean DNA with comparable length, whereas most nick-column-based mini-preparation methods yield shorter DNA with many single-strand nicks.

B. DNA digestion

1 Digest 5 μg plant DNA in 200 μl appropriate restriction endonuclease buffer (61) for at least 2 h using an enzyme (100 U), which has either no or only a single cleavage site within the T-DNA.

Protocol 4 continued

2 After testing 10 μl aliquots by gel electrophoresis, extract the samples twice with phenol/chloroform/isoamylalcohol, add 20 μl of 3 M sodium-acetate (pH 6.0), and precipitate the DNA with 150 μl isopropanol on ice for 30 min.

3 Pellet the DNA by centrifugation in a microfuge for 5 min, wash with 70% ethanol, dry briefly, and dissolve in 25 μl sterile water.

C. Ligation of DNA

1 Self-ligate 1 μg digested DNA in 200 μl T4 DNA ligase buffer containing 10% polyethylene glycol (PEG 4000) at 15 °C for 8 h.

2 Extract the samples twice with phenol/chloroform/isoamylalcohol and precipitate the DNA as described above (Protocol 4A9). Digest the samples with an enzyme that cleaves once the T-DNA segment used for self-ligation, but has a cleavage site frequency in the *Arabidopsis* genomic DNA less than 1 for every 50 kb (i.e. *Sma* I, *Sal* I etc.).

3 Linearization of the self-ligated DNA is, however, not essential for iPCR amplification, thus this step may be omitted.

D. Design of PCR primers

The primer design depends on whether the enzyme used in step B cleaves within the T-DNA.

If the enzyme does not cleave in the T-DNA:

1 The self-ligated plant DNA sequence can be PCR amplified using two T-DNA primers that are oriented toward the left and right termini of the T-DNA.

2 Because deletions are often generated at the left border during T-DNA integration, design the left border primer such that it is located at least 100 bp upstream of the left 25 bp border repeat.

3 In contrast, deletions at the right T-DNA end are rare, thus the second primer can safely be placed 20–50 bp upstream of the right 25 bp repeat.

4 It is advisable to design nested primers placed at different distances from the T-DNA ends to ensure the success of iPCR and increase the amount of information obtained by subsequent sequencing of the rescued plant DNA segment using the T-DNA end-primers.

If the enzyme cleaves the T-DNA:

1 Design two primers such that they face the enzyme cleavage site from 5′ and 3′ orientation.

2 Use these primers in pairwise combinations with the T-DNA end-primers for PCR amplification and then independently for PCR sequencing.

E. Polymerase chain reactions

1 The iPCR reactions are performed using either Elongase (GibcoBRL) or Takara LA-PCR (Boehringer Ingelheim) enzyme mixes as recommended by the suppliers.

Protocol 4 continued

2 Amplify 0.5 μg template DNA in 50 μl Elongase PCR buffer containing 1–2.5U enzyme and 0.2 μM of each primer.

3 Following denaturation at 95 °C, run 35 cycles (94 °C for 30 s, 65 °C for 30 s, 68 °C for 8 min) followed by elongation at 68 °C for 10 min.

4 Test 2 μl aliquot from each PCR reaction by gel electrophoresis.

5 In rare cases, when no amplification of defined fragments is obtained, perform a new PCR with the nested primers (see *Protocol 4D*) using either fresh template DNA or 1 μl from a 500-fold diluted PCR reaction mix.

6 Purify the PCR amplified DNA fragments from agarose gel using electroelution (60).

7 Clean the DNA by phenol/chloroform extraction and precipitate with isopropanol as described above (Protocol 4A9–4A11)

8 Sequence the DNA fragments using an ABI Prism-Dye Terminator Cycle Sequencing kit according to the protocol of manufacturer.

F. Sequence analysis

1 To find possible gene hits, analyse the obtained plant DNA sequences using the BLASTN and BLASTX programs by performing searches in the non-redundant, EST and *Saccharomyces* databases (http://www.ncbi. nlm.nih.gov).

2 Alternatively run searches using, for example, databases at http:// genome-www. stanford. edu/Arabidopsis/, or http://www.tigr.org/tbd/.

3 After translating the DNA to protein sequences it is possible to search for motifs at http://coot.embl-heidelberg.de/ SMART/ or http://www.motif.genome.ad.jp/. Other useful sequence analysis facilities are listed at http://www.columbia.edu/~ej67/dtb_lst.htm, http://www.genome.ad.jp/, http://www.infobiogen.fr/services/dbcat/ and http:// www.molbio.net/genomics.html.

References

1. Zambryski, P. C. (1992). *Annu. Rev. Plant Phys. Plant Mol. Biol.*, **43**, 465.
2. Koncz, C., Németh, K., Rédei, G. P. and Schell, J. (1994). In *Homologous Recombination in Plants* (ed. J. Paszkowski), pp. 167–189. Kluwer Academic Press, Dordrecht.
3. Koncz, C., Martini, N., Mayerhofer, N., Koncz-Kálmán, Z., Körber, H., Rédei, G. P. and Schell, J. (1989). *Proc. Natl Acad. Sci. USA*, **86**, 8467.
4. Azpiroz-Leehan, R. and Feldmann, K. A. (1997). Trends Genet. **13**, 152.
5. Koncz, C., Németh, K., Rédei, G. P. and Schell, J. (1992). *Plant Mol. Biol.*, **20**, 963.
6. Topping, J. F. and Lindsey, K. (1995). *Transgenic Res.*, **4**, 291.
7. Kakimoto, T. (1996). *Science*, **274**, 982.
8. Koncz, C., Martini, N., Szabados, L., Hrouda, M., Bachmair, A. and Schell, J. (1994). In *Plant Molecular Biology Manual* (ed. S. Gelvin, and R. Schilperoort B), Vol. B2, pp. 1–22. Kluwer Academic Press, Dordrect.
9. Depicker, A. G., Jacobs, A. M. and Van Montagu, M. C. (1988). *Plant Cell Rep.*, **7**, 63.
10. Czakó, M. and Márton, L. (1994). *Plant Physiol.*, **104**, 1067.
11. Kobayashi, T., Hisajima, S., Stougaard, J. and Ichikawa, H. (1995). *Jap. J. Genet.*, **70**, 409.

12. Osborne, B. I., Wirtz, U. and Baker, B. (1995). *Plant J.*, **7**, 687.
13. Kilby, N. J., Davies, G. J., Snaith, M. R. and Murray, J. A. H. (1995). *Plant J.*, **8**, 637.
14. Koncz, C., Schell, J. and Rédei, G.P. (1992). In *Methods in Arabidopsis Research* (ed. C. Koncz, N-H. Chua, and J. Schell), pp. 224–273. World Scientific, Singapore.
15. Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994). *Plant J.*, **6**, 271.
16. Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T. and Kumashiro, T. (1996). *Nature Biotechnol.*, **14**, 745.
17. Tingay, S., McElroy, D., Kalla, R., Fieg, S., Wang, M. B., Thornton, S. and Brettell, R. (1997). *Plant J.*, **11**, 1369.
18. Cheng, M., Fry, J. E., Pang, S., Zhou, H., Hironaka, C. M., Duncan, D. R., Conner, T. W. and Wan, Y. (1997). *Plant Physiol.*, **115**, 971.
19. Enriquezobregon, G. A., Vazquezpadron, R. I., Prietosamsonov, D. L., Delariva, G. A. and Selmanhousein, G. (1998). *Planta*, **206**, 20.
20. Bundock, P., den Dulk-Ras, A., Beijersbergen, A. and Hooykaas, P. J. J. (1995). *EMBO J.*, **14**, 3206.
21. de Groot, M. J., Bundock, P., Hooykaas, P. J. and Beijersbergen, A. G. (1998). *Nature Biotechnology*, **16**, 839.
22. Ziemienowicz, A., Görlich, D., Lanka, E., Hohn, B. and Rossi, L. (1999). *Proc. Natl Acad. Sci. USA*, **96**, 3729.
23. Mathur, J., Szabados, L., Schaefer, S., Grunenberg, B., Lossow, A., Jonas-Straube, E., Schell, J., Koncz, C. and Koncz-Kálmán, Z. (1998). *Plant J.*, **13**, 707.
24. Feldmann, K. A. and Marks, M. D. (1987). *Mol. Gen. Genet.*, **208**, 1.
25. Feldmann, K. A. (1991). *Plant J.*, **1**, 71–82.
26. Bechtold, N., Ellis, J. and Pelletier, G. (1993). *C. Roy. Acad. Sci.*, **316**, 1188.
27. Clough, S. J. and Bent, A. F. (1998). *Plant J.*, **16**, 735.
28. Koncz, C. and Schell, J. (1986). *Mol. Gen. Genet.*, **204**, 383.
29. Koncz, C., Chua, N-H. and Schell, J. (1992). *Methods in Arabidopsis Research*. World Scientific, Singapore
30. Meyerowitz, E. M. and Somerville, C. R. (1994). *Arabidopsis*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
31. Li, S. L. and Rédei, G. P. (1969). *Rad. Bot.*, **9**, 125.
32. Peirson, B. N., Owen, H. A., Feldmann, K. A. and Makaroff, C. A. (1996). *Sexual Plant Rep.*, **9**, 1.
33. Bonhomme, S., Horlow, C., Vezon, D., de Laissardiere, S., Guyon, A., Ferault, M., Marchand, M., Bechtold, N. and Pelletier, G. (1998). *Mol. Gen. Genet.*, **260**, 444.
34. Caiping, H. and Mascarenhas, J. P. (1998). *Sexual Plant Rep.* **11**, 199.
35. Howden, R., Park, S. K., Moore, J. M., Orme, J., Grossniklaus, U. and Twell, D. (1998). *Genetics*, **149**, 621.
36. Sanders, P. M., Bui, A. Q., Weterings, K., McIntire, K. N., Hsu, Y.-C., Lee, P. Y., Truong, M. T., Beals, T. P. and Goldberg, R. B. (1999). *Sexual Plant Rep.*, **11**, 297.
37. Zhang, J. Z. and Somerville, C. R. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 7349.
38. Uwer, U., Willmitzer, L. and Altmann, T. (1998). *Plant Cell*, **10**, 1277.
39. Albert, S., Després, B., Guilleminot, J., Bechtold, N., Pelletier, G., Delseney, M. and Devic, M. (1999). *Plant J.*, **17**, 169.
40. Oppenheimer, D. G., Pollock, M. A., Vacok, J., Szymanski, D. B., Ericson, B., Feldmann, K. and Marks, D. M. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 6261.
41. Wang, H., Lockwood, S. K., Hoeltzel, M. F. and Schiefelbein, J. W. (1997). *Genes Devel.*, **11**, 799.
42. Barthels, N., van der Lee, F. M., Klap, J., Goddijn, O. J. M., Karimi, M., Puzio, P., Grundler, F. M. W., Ohl, S. A., Lindsey, K., Roberstson, L., Robertson, W. M., Van Montagu, M., Gheysen, G. and Sijmons, P. C. (1997). *Plant Cell*, **9**, 2119.

43. Favery, B., Lecomte, P., Gil, N., Bechtold, N., Bouchez, D., Dalmasso, A. and Abad, P. (1998). *EMBO J.*, **17**, 6799.
44. Revenkova, E., Masson, J., Koncz, C., Afsar, K., Jakovleva, L. and Paszkowski, J. (1999). *EMBO J.*, **18**, 490.
45. Thion, L., Mazars, C., Nacry, P., Bouchez, D., Moreau, M., Ranjeva, R. and Thuleau, P. (1998). *Plant J.*, **13**, 603.
46. Gaymard, F., Pilot, G., Lacombe, B., Bouchez, D., Bruneau, D., Boucherez, J., Michaux-Ferrière, N., Thibaud, J.-B. and Sentenac, H. (1998). *EMBO J.*, **94**, 647.
47. Nam, J., Mysore, K. S., Zheng, C., Knue, M. K., Matthysse, A. G. and Gelvin, S. B. (1999). *Mol. Gen. Genet.*, **261**, 429.
48. Ballinger, D. G. and Benzer, S. (1989). *Proc. Natl. Acad. Sci. USA*, **86**, 9402.
49. Kaiser, K. and Goodwin, S. F. (1990). *Proc. Natl. Acad. Sci. USA*, **87**, 1686.
50. Rushforth, A. M., Saari, B. and Anderson, P. (1993). *Mol. Cell. Biol.*, **13**, 902.
51. Zwaal, R. R., Broeks, A., van Meurs, J., Groenen, J. T. M. and Plasterk, R. H. A. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 7431.
52. Koes, R., Souer, E., van Houwelingen, A., Mur, L., Spelt, C., Quattrocchio, F., Wing, J., Oppedijk, B., Ahmed, S., Maes, T., Gerats, T., Hoogeveen, P., Meesters, M., Kloos, D. and Mol, J. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 8149.
53. McKinney, E. C., Ali, N., Traut, A., Feldmann, K. A., Belostotsky, D. A., McDowell, J. M. and Meagher, R. B. (1995). *Plant J.*, **8**, 613.
54. Krysan, P. J., Young, J. C., Tax, F. and Sussman, M. R. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 8145.
55. Baumann, E., Lewald, J., Saedler, H., Schulz, B. and Wisman, E. (1998). *Theor. Appl. Genet.*, **97**, 729.
56. Souer, E., Quattrocchio, F., de Vetten, N., Mol, J. and Koes, R. (1995). *Plant J.* **7**, 677.
57. Winkler, R. G., Frank, M. R., Galbraith, D. W., Feyereisen, R. and Feldmann, K. A. (1998). *Plant Physiol.*, **118**, 743.
58. Liu, Y-G., Mitsukawa, N., Oosumi, T. and Whittier, R. F. (1995). *Plant J.*, **8**, 457.
59. Frey, M., Stettner, C. and Gierl, A. (1998). *Plant J.*, **13**, 717.
60. Dellaporta, S. L., Wood, J. and Hicks, J. B. (1983). *Plant Mol. Biol. Rep.*, **1**, 19.
61. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor.