EXOTIC Exon Trapping Insert Consortium

The EXOTIC project is funded through the Fifth Framework programme of the EU for Plant Biotechnology. The EXOTIC project initiates a large-scale programme aimed at determining the expression patterns of approximately 5000 genes from Arabidopsis. These expression patterns reveal one of the important facets of gene function that can be linked to others, such as phenotypes from loss-of-function and mis-expression mutants, and predictions based on protein sequence and structure, to reveal an holistic and predictive view of the cellular roles of gene products. This approach may reveal aspects of the functions of essential genes that are not amenable to genetic analysis.

The EXOTIC Handbook was conceived as an aid to participants within the consortium and to researchers and Plant Biotechnologist. The Handbook gives an overview of Gene-Trapping in Arabidopsis, using the *Ds* gene-traps designed by Rob Martienssen and colleagues at the Cold Spring Harbor Laboratory, and a series of detailed protocols. The Handbook will shortly be available via the web and will be regularly updated and revised to give up-to-date protocols and details of new constructs currently in development.

I hope you find the Handbook a useful aid in your research and will be glad to receive your feedback on changes and improvements. mailto:jonathan.clarke@bbsrc.ac.uk

Jonathan Clarke Ph.D. John Innes Centre, March 2000.

Notes for Second Edition:
To complement the first Gene Trap Workshop, held at the JIC from January 29-31, 2001 the Handbook has been revised and updated. I have included more images of the phenotypes observed during the selection of F2 transposant, and extended the range of protocols to cover the preparation of DNA, diagnostic PCRs to eliminate escapes from the F2 selection, and all our greenhouse protocols.
Thanks to all those who helped in revising the protocols, especially Sally Langham, Beth McCullagh, Lesley Phillips, Bee Bowles and Paul Langham at ATIS.

Jonathan Clarke Ph.D., John Innes Centre, June 2001

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Objectives and Expected Achievements

The goal of this project is to initiate a large -scale project aimed at determining the expression patterns of approximately 5,000 genes from *Arabidopsis thaliana* (Arabidopsis). The propensity of transposons to jump to linked sites from a launching pad will be exploited on a large scale for the first time to generate a new spectrum of gene disruptions. The Ds transposable element has been modified to detect plant gene expression patterns by incorporating a splice acceptor in three reading frames with a GUS reporter gene. The GUS reporter sensitively detects very low levels of gene expression, and is used widely to define the cellular specificity and inducibility of plant gene expression patterns.

Defining the expression patterns of genes reveals one of the important facets of gene function. Linked to others data sources, such as phenotypes from loss-of-function and mis-expression mutants, and predictions based on protein sequence and structure, expression patterns reveal an holistic and predictive view of the cellular roles of gene products. Strategies involving the definition of gene expression patterns have revealed the functions of both individuals and family members more efficiently, and lead to phenotypes in the heterozygous state that reveal the function of genes necessary for cell viability. This approach may reveal aspects of the functions of essential genes that are not amenable to genetic analysis. Furthermore, patterns of gene expression directed by promoter sequences reveal the information content of non-coding regulatory regions of the genome. This proposal will form an important component of world-wide activities aimed at determining the functions of plant genes using these complementary approaches, as it offers a new strategy for obtaining genome-wide coverage of a large number of insertions.

A systematic approach to revealing phenotypes and describing gene expression patterns will be carried out. First, an established system for generating Ds insertions at new positions, both linked and unlinked to the launching pad will be used to initiate large-scale mutagenesis. This activity will be carried out by all partners to generate a large population of insertions at new locations throughout the genome. Second, an improved method for generating translational fusions will be tested and utilized in an "unlinked" strategy. GUS expression patterns in transposant populations will be screened in particular tissues and in several growth conditions, according to the interests and expertise of the participants. Any phenotypes associated with homozygous insertions will be assessed in specific screens adapted to the screens, will be described using a controlled vocabulary. The data will be pooled and a specific database made that contains images of expression patterns, sequence of the disrupted gene and position of insertion in the gene, and possible mutant phenotypes and a description of the expression patterns and phenotypes. This database will be an intrinsic part of the new Arabidopsis database and will also be linked to, and be an annotated component of, the major Arabidopsis sequence databases.

Summary of Objectives

- Generation of a new population of approximately 50,000 transposon insertions in the Arabidopsis genome, with a significant proportion of insertions in sequenced regions.
- Approximately 10-20% of the insertions will generate translational fusions with the GUS reporter gene, and many of these will be enzymatically active.
- Expression patterns will be assessed in several tissue types and in response to several treatments and environmental stimuli.
- Further studies of possible subcellular localisation of the GUS gene fusion are possible.
- The transposon insertion sites in lines expressing GUS will be sequenced to relate expression and potential phenotype to a disrupted gene.
- A catalogue of expression patterns and any associated phenotypes caused by loss of gene function will be made and placed in a searchable database, and linked to genome sequence and any quantitative microarray data generated.
- The information content of non-coding regulatory regions of the genome is revealed for many genes.
- A catalog of promoter expression patterns will be generated.

Gene Trap Transposon Mutagenesis in *Arabidopsis*

(adapted from "Enhancer and Gene trap transposon mutagenesis in Arabidopsis", R. Martienssen and P Springer, 1998 in "Insertional Mutagenesis: a practical approach" Oxford University Press (G Coupland, ed.)

Gene traps are reporter gene constructs that can respond to cis-acting transcriptional signals when integrated into chromosomal DNA. Insertional mutagenesis using these "traps" involves generating a large number of individuals that have the reporter gene integrated at different sites throughout the genome. Their progeny are collected and examined for expression of the reporter gene and mutant phenotypes caused by insertion. In lines in which the reporter gene is inserted within or near a chromosomal gene, reporter gene expression mimics that the chromosomal gene. In the last few years gene traps have been extensively exploited in *Drosophila* and in mouse developmental genetics, and several modifications have been made to the basic systems. In plants, gene traps have taken a number of different forms, depending on the reporter gene construct and the vector used for insertional mutagenesis. T-DNA and transposons have both been used to introduce promoter traps into *Arabidopsis.* In this summary, we will describe the gene trap transposon system that we, and our colleagues have utilizing in *Arabidopsis.*

Gene trap transposons

Gene trap reporter genes (promoter traps and exon traps) have no promoter, so that reporter gene expression can occur only when the reporter gene inserts within a transcribed chromosomal gene, creating a transcriptional fusion. Our system uses gene trap reporters, and is based on the Maize *Ac* and *Ds* transposable elements (Figure 1). The *Ds* elements carry the β-glucuronidase (GUS) gene as a reporter and the Neomycin phosphotransferase (NPTII) gene (conferring resistance to kanamycin) as a selectable marker. The *Ds* element used in the gene trap has a multiple splice acceptor fused to the GUS gene. Random insertions of the *Ds* element throughout the genome allow us to detect chromosomal gene expression through the activation of the GUS gene.

Mutagenesis

Ds elements are transactivated by crossing to transgenic plants that provide a source of transposase, namely an immobilized *Ac* element (Figure 1). The Indole acetamide hydrolase (IAAH) gene has been incorporated in the T-DNAs carrying both the *Ds* elements and the *Ac* element. Selection against the IAAH gene using the herbicide analog napthalene acetamide (NAM), and for the *Ds* element using kanamycin allows the recovery of transposition events that have lost (by recombination) the donor locus, and thereby enriches for unlinked transposition events. Since we also select against the *Ac* element, the insertion is immediately stabilized.

Mutagenesis is initiated by crossing plants homozygous for one of the *Ds* elements to plants containing the Ac transposase gene (Protocol 1; Figure 2A & B). The resulting F1 seed are planted, and the plants are allowed to self-pollinate. The F2 seed are harvested from each individual F1 plant, and plated on media containing kanamycin (selects for the *Ds* element) and NAM (selects against both T-DNAs) (Protocol 2). The double resistant F2 seedlings (called transposants) contain a transposed *Ds* element (Figure 3). The NamR KanR seedlings are transplanted to soil, and allowed to self-fertilize. F3 seed

are collected, and grown, and the resulting plants are stained for GUS activity and examined for mutant phenotypes.

For detailed methods see protocols1, 2 & 3.

Screening transposant lines

Transposant lines can be screened for mutant phenotypes caused by insertion, and for patterns of reporter gene expression. In some cases, it may be beneficial to stain transposants first, and then use the staining pattern to guide phenotypic examination. Although only a fraction of insertions into genes will result in reporter gene expression, the remaining lines without reporter gene provide a source of gene disruptions. Individual F2 plants may be either homozygous or heterozygous for the transposed *Ds* element. Any phenotype associated with the insertion will be present in every F3 plant (in the case of a F2 homozygote) or segregate 3:1 normal to mutant (if the F2 plant was heterozygous and the mutation is recessive). Segregation ratios substantially more than 3:1 may indicate that the mutation arose after transposition and is not associated with the insertion.

Lethal insertions

Lethal insertions can be scored most conveniently in F2 transposants, by opening developing siliques and scoring unfertilized ovules and colorless embryos. Lethal situations arise at a frequency of about 4%. Lethals should be carefully tested for heritability and association with the *Ds* insertion by plating F3 seed on kanamycin (the resistance gene within the *Ds* element). If the insertion is responsible for the lethal phenotype, all resistant F3 plants (which are heterozygous for the insertion) should give rise to plants with semi-sterile siliques or defective embryos. Lethality in the gametophyte or in the embryo should result in poor transmission of the kanamycin resistant trait, with ratios of between 1:1 and 2:1 resistant to sensitive F3 seed.

Screening for reporter gene expression

Screening for reporter gene expression patterns should be done wherever possible in heterozygous plants or in segregating families that include phenotypically normal individuals (Protocol 3). This is because homozygosity for the insertion can affect report gene expression patterns in many cases. It is wise to examine several individual plants, so staining in the F3 is recommended. Potassium ferricyanide is included in the staining reaction in order to catalyze dimerization of the indigo monomer, which is the product of the glucuronidase reaction. The colorless monomer is soluble, while the dimer is not, resulting in a blue precipitate at the site of enzyme activity. In the absence of at least 1.5mM ferricyanide, diffusion of the indigo monomer results in artefactual staining patterns. Unfortunately, ferricyanide ions also inhibit the GUS enzyme at these concentrations. This inhibition can be partially overcome by using long incubation times for weaker staining patterns. Clearing of the tissue following staining is most simply and gently accomplished using 70% ethanol, which is adequate for all tissues. However, other more drastic clearing methods can be employed, but need to be carefully controlled with respect to re-dissolving the stain and damaging the tissue. Cleared tissue is mounted in glycerol and viewed with Nomarski optics. An example of a line in which the reporter gene is expressed in roots is shown in Figure 4.

Molecular analysis of transposants

Each transposant line that has a staining pattern or phenotype of interest can be analyzed using molecular methods to determine the location of the transposed element in the *Arabidopsis* genome. Flanking genomic DNA or cDNA corresponding to the gene nearest the insertion site can be rapidly obtained by PCR amplification. The resulting products can be sequenced directly, or used as hybridization probes for further analysis.

Insertions into T-DNA

Although most insertions are randomly distributed around the genome, about 5 percent of transposants have *Ds* insertions into the IAAH gene on the T-DNA. These insertions disrupt the IAAH gene resulting in resistance to the negative marker NAM. IAAH insertions typically have strong ubiquitous reporter gene expression in seedling tissues and can often be discarded on the basis of this expression pattern or on the basis of further molecular analysis (see below). However, if this is not possible, flanking DNA can be sequenced as described below.

Amplification of flanking genomic DNA

Chromosomal sequences flanking gene trap insertions can be amplified using I (inverse) PCR (Protocol 4A) and TAIL (thermal asymmetric Inter-laced) PCR (Protocol 4B) using standard protocols and primers from the *Ds* element. These PCR products can be sequenced directly after purification on spin columns or gels by cycle sequencing using dye terminators. Each of these methods depends on the fortuitous location of a primer sequence or restriction site close to the insertion, and consequently is only successful about 50% of the time for a given primer/enzyme combination. It is wise, therefore, to use several prime combinations or several approaches when attempting to amplify a given insertion site.

Amplification of cDNA from gene trap fusions

Gene trap insertions result in transcriptional fusions between the reporter gene and the chromosomal gene into which it is inserted. Consequently, flanking sequences can be amplified by 5'RACE PCR using RNA isolated from the gene trap line (Protocol 5). This is useful when the transposon is inserted into a large intron, or when multiple introns make chromosomal sequence hard to interpret. RACE PCR products can also be sequenced directly, except that alternate splicing will result in mixed sequence reads in many cases. In these cases, sub-cloning will be required.

In either case, it is wise to confirm the assignment of a given sequence to a give line by hybridization of the products to genomic Southern blots, or by PCR using specific primers derived from the sequence.

Genetic analysis of transposants

In many cases, insertion of a transposon will be associated with a mutant phenotype. Spontaneous mutants arise at a surprisingly high frequency in transposon lines, and it is important to determine whether the transposon is responsible for any observed phenotype.

- First, the insertion should be mapped genetically, relative to the mutant phenotype and to other genetic and molecular markers.
- Second, the transposon at the locus in question should be re-mobilized. The ability to remobilize transposons so that they leave one locus and re-insert elsewhere allows the construction of the allelic series.

The most important application of the second approach is in the analysis of revertants. The restoration of a wild-type phenotype when the transposon excises provides strong evidence that the transposon was responsible for the mutation. A second application is in the disruption of nearby genes. These procedures are described below.

Mapping

Mapping of transposed elements can be most readily accomplished by amplifying flanking DNA (see protocol 4A & B) and aligning with the genomic sequence of *Arabidopsis* by BLAST searches. If no alignment is identified the resulting PCR product may be used as a probes to hybridize to anchored libraries, or to Southern blots of DNA from mapping populations such as recombinant inbred lines.

Phenotypically, the DsG transposons each carry a kanamycin-resistance gene. This means that each insertion can be mapped with respect to any associated mutant genotype, as well as to previously mapped phenotypic and molecular markers. Plants heterozygous for the insertion are outcrossed to wild-type plants, and F1 progeny are sowed to self-pollinate. F2 families are sown once to screen for any mutant phenotype, and again to screen for kanamycin resistance. Mutations that are caused by the insertion will only be found in F2 families with kanamycin resistant progeny. If some kanamycin resistant families have no mutant progeny, this might indicate poor penetrance of the mutation, or the presence of a second insertion elsewhere in the genome. If the insertion causes a lethal mutation, the ratio of kanamycin resistant to kanamycin sensitive seedlings should be less than 3:1 on self-pollination of a heterozygous F1 plant. The ratio will be 2:1 for an embryo lethal, and 1:1 for a gametophyte lethal.

By using a wild-type parent from a different ecotype (Columbia), F2 seed can be used to map the insertion. This is accomplished by preparing DNA from pooled F2 seedlings from kanamycin resistant and kanamycin sensitive families, and screening the DNA samples with PCR-based polymorphic markers.

Reversion

Insertion of a *Ds* transposon results in the duplication of 8 bp of target sequence immediately flanking the insertion site. When *Ds* excises, the target duplication is partially removed, resulting in small

insertions and deletions at the original locus. If the *Ds* is inserted into the coding region of the gene, only those events that restore the reading frame and result in a functional protein will revert the mutant phenotype back to wild-type. In contrast, almost all reversions from non-essential sequences such as introns will result in reversion of the mutant phenotype.

Reversion is accomplished by crossing mutant plants to transgenic plants that carry the transposase gene (Protocol 6). The resulting F1 plants are then planted and allowed to self-pollinate. The F2 progeny will now include mutants that carry the transposase gene. The transposon responsible for the mutant phenotype will excise in these plants resulting in somatic sectors of tissue that have lost the transposon. If these plants are mosaics for the mutant phenotype, this is good evidence that the phenotype can be reverted by transposase. More importantly, a proportion of the F3 progeny of these plants should be wild-type, in contrast to the progeny of mutants that do not carry the transposase gene, which should be true-breeding mutant. Revertant alleles can be amplified from wild-type progeny using primers that flank the insertion site. These products can then be sequence to determine the nature of the reversion event. In the special case of lethal mutations, reversion can be observed in the F1 plants themselves. Most of the siliques on these plants should be semi-sterile, or carry dead seed, depending on whether the mutation is lethal at the gametophytic or the embryonic stage. However, reversion early in development will result in normal, fully fertile siliques provided reversion occurs early enough to detect revertant branches.

Reinsertion

There are many circumstances when reinsertion of a transposon by short-range transposition is advantageous. For example, gene trap insertions may not disrupt gene function, as insertions within introns may be spliced from the RNA transcript without phenotypic effect. In these cases, it can be useful to obtain a secondary insertion into the nearest exon by inducing a short-range transposition. A high proportion of transpositions of *Ds* are to closely linked sites when these transpositions are not counter-selected (see introduction). A protocol for re-mobilization is given below.

In brief, the transposon is re-mobilized by crossing to transposase, and is then stabilized by selecting against the transposase gene in the next generation (Figure 4). The parental transposon is not selected against by this procedure, and so a large proportion (more than half) of the resulting plants will still have the transposon inserted at the original location. Those plants that have new insertions therefore need to be identified molecularly, phenotypically, or by staining for reporter gene expressions. In our experience, a collection of 2,000 plants selected in this manner will carry between 500 and 1,000 new transpositions. About 20% of these will be within 100kb. This should be sufficient to saturate the nearby region with new insertions (C. Yordan and R. Martienssen, unpubl. Observations).

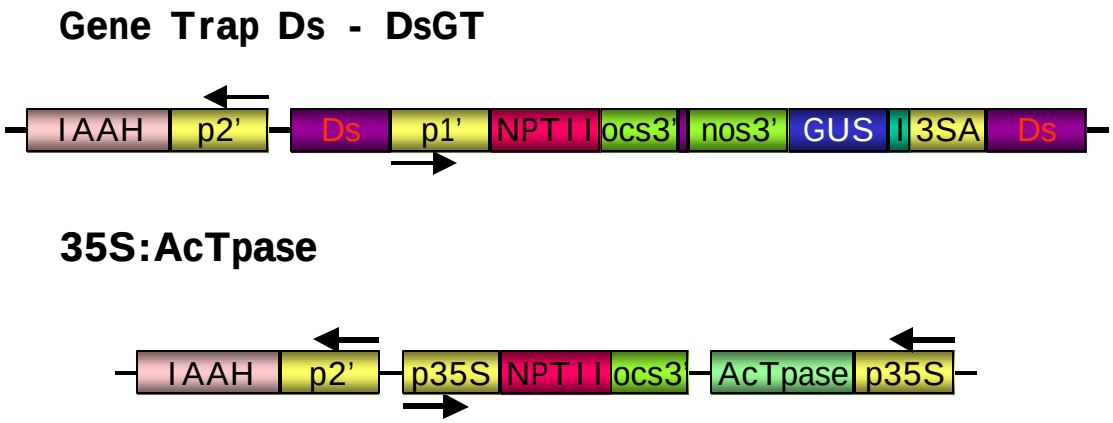

Figure 1. Schematic Transposon constructs. Schematic diagrams of the Ac and Ds elements are shown incorporated into T-DNA. IAAH, indole acetamide hydrolase gene (confers sensitivity to naphthalene acetamide, NAM); NPTII, neomycin phosphotransferase gene (confers resistance to kanamycin); GUS, b-glucuronidase reporter gene; 3SA, triple splice acceptor; LB, RB left and right T-DNA borders; 1', 2' T-DNA promoters; ocs 3', nos 3' T-DNA transcription terminators; Δ35S, minimal promoter (-46 truncation of viral 35S promoter). DsG is the Gene trap transposon.

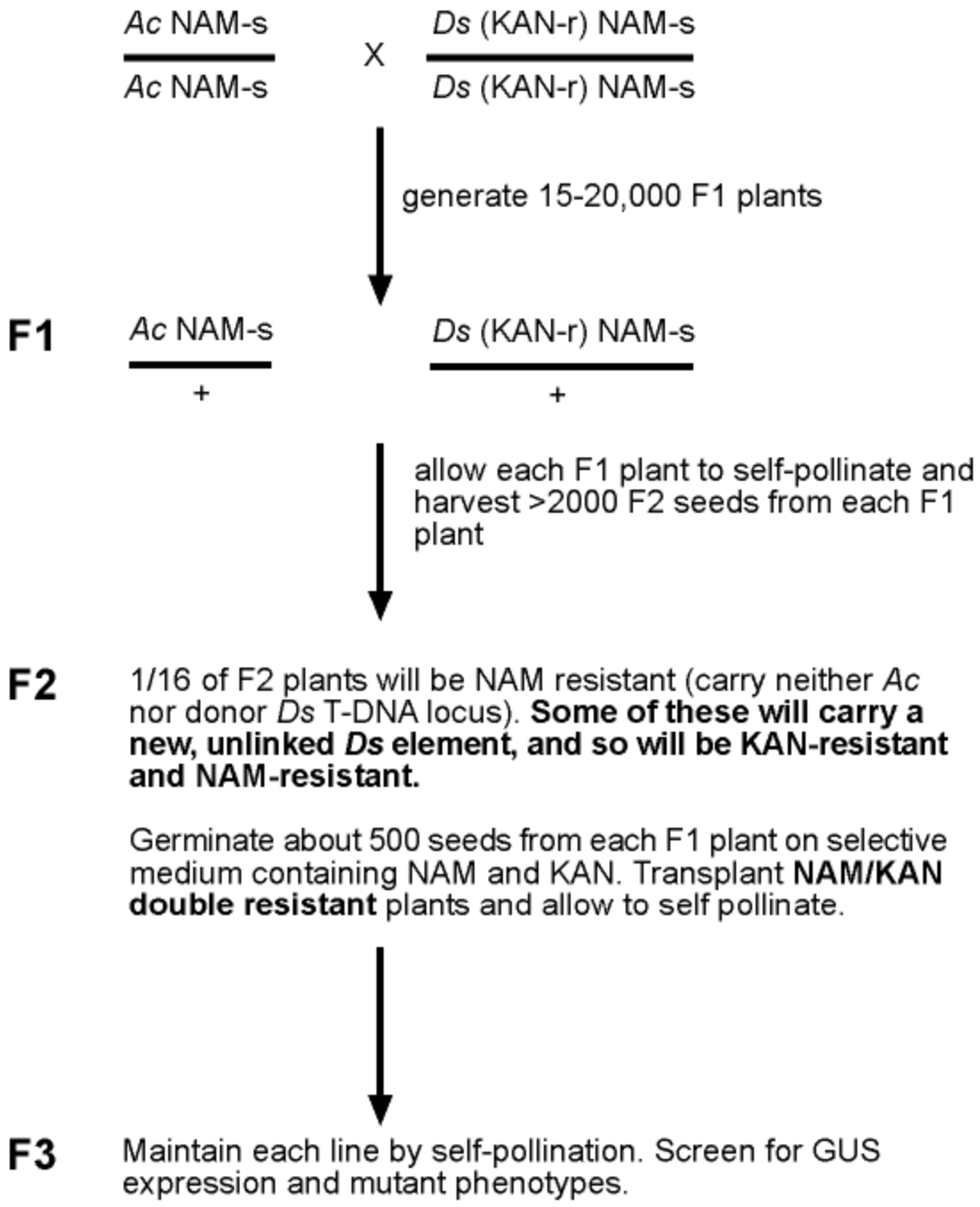

Figure 2A. Mobilization scheme for unlinked transpositions. Mutagenesis crosses and the expected frequency of mutagenized progeny are indicated in the scheme (Protocols 1 and 2). NAM, naphthalene acetamide; IAAH indole acetamide hydrolase; kan, kanamycin.

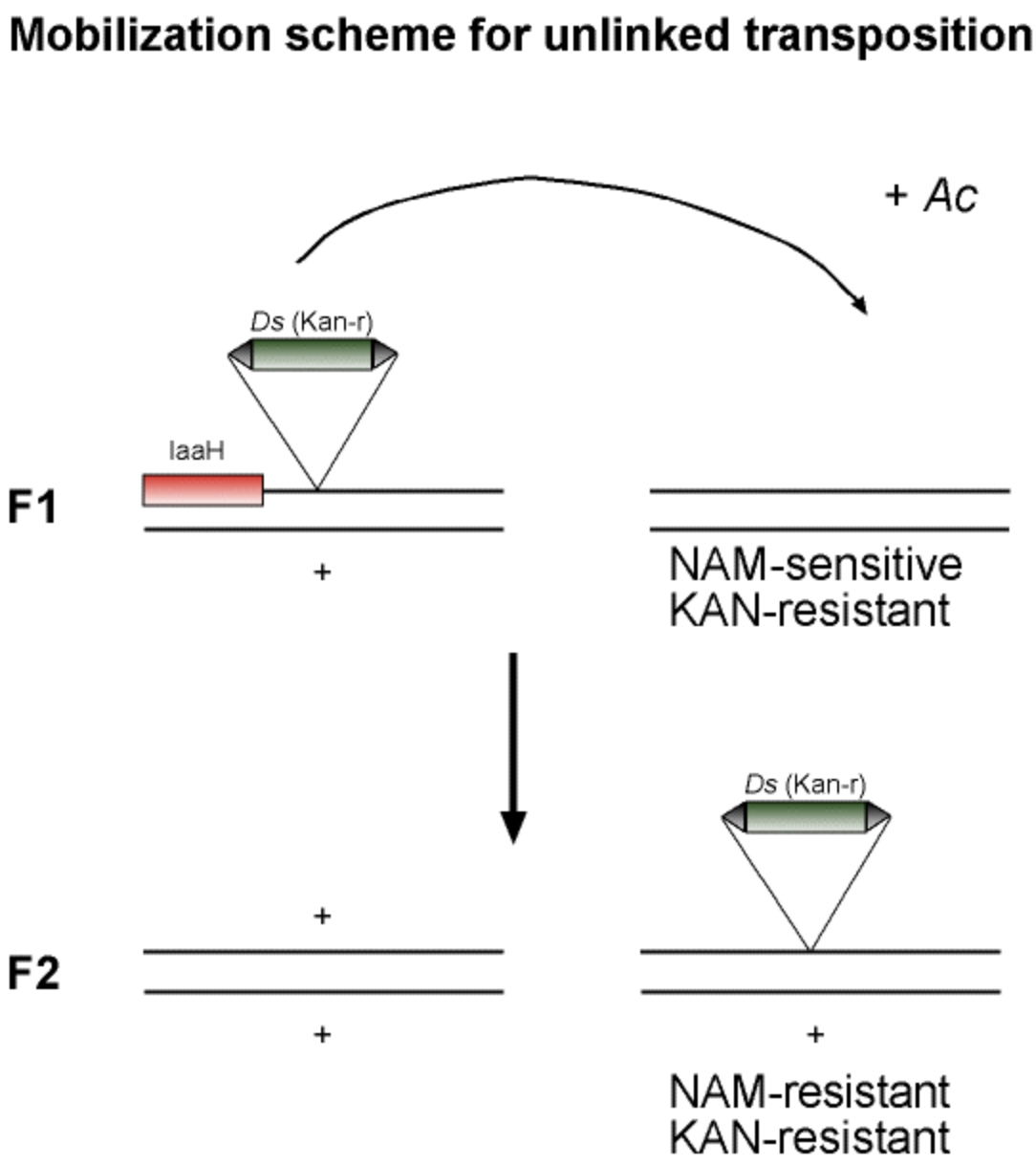

Figure 2B. Mobilization scheme for unlinked transpositions. Mutagenesis crosses and the expected frequency of mutagenized progeny are indicated in the scheme (Protocols 1 and 2). NAM, naphthalene acetamide; IAAH indole acetamide hydrolase; kan, kanamycin.

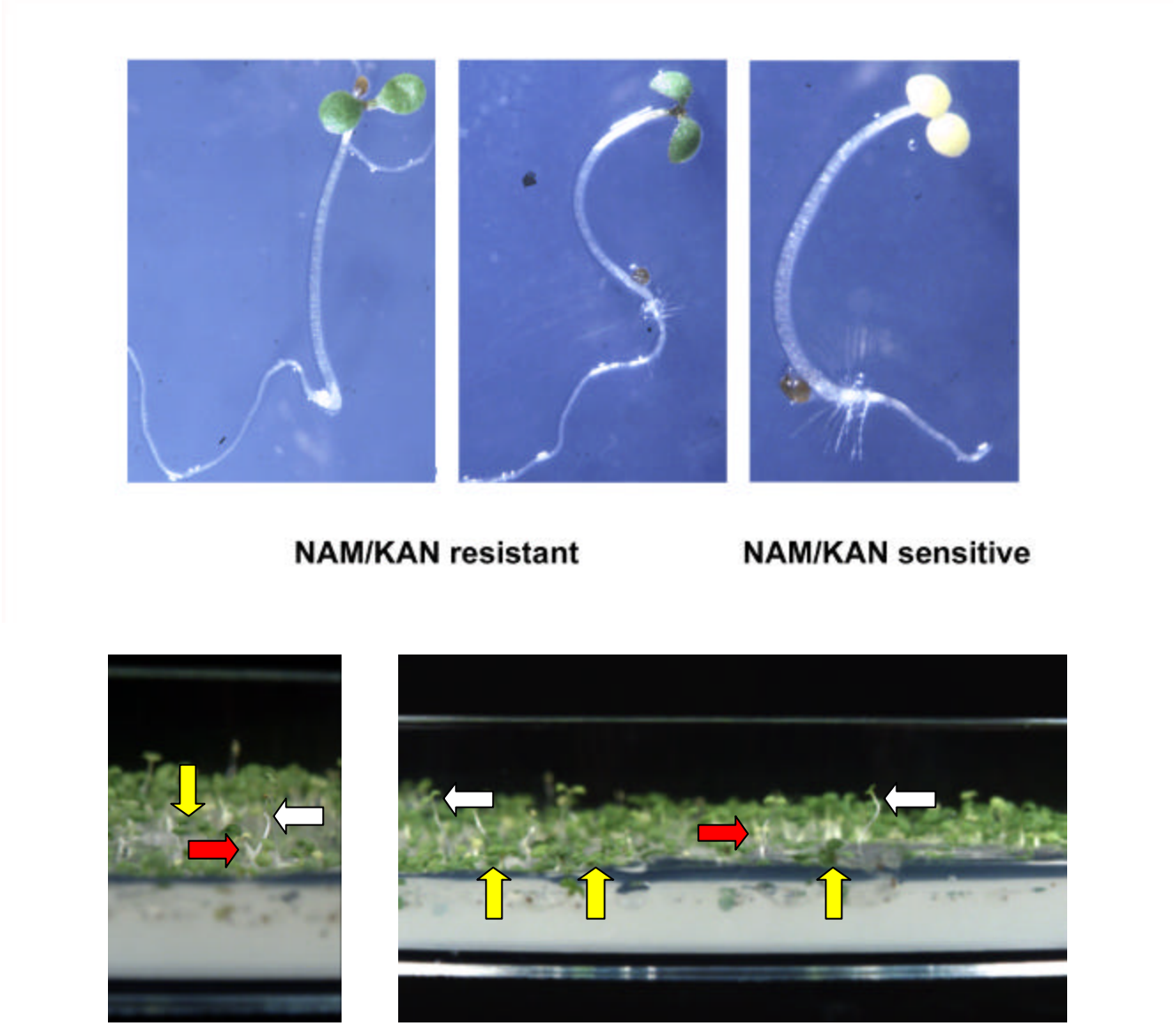

Figure 3. NAM / KAN double selection. NAM / KAN double resistant seedlings are elongated with green cotyledons and normal roots ⇨ . Sensitive seedlings are elongated but have white cotyledons ➡ and short roots or remain as green non elongated seedlings ➡ .

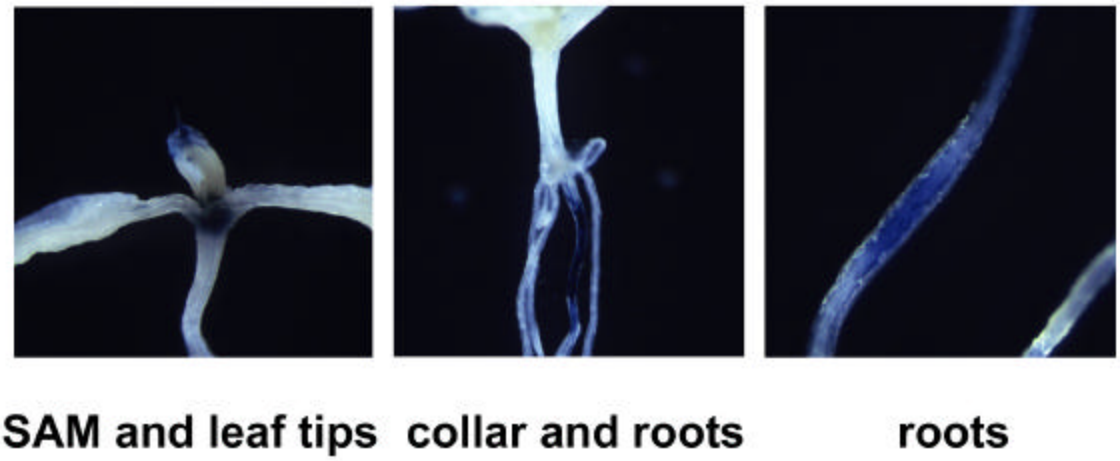

Figure 4. Gene trap reporter gene expression in SAM and leaf tips, collar and root, and root. Tissue samples were stained and photographed according to Protocol 3

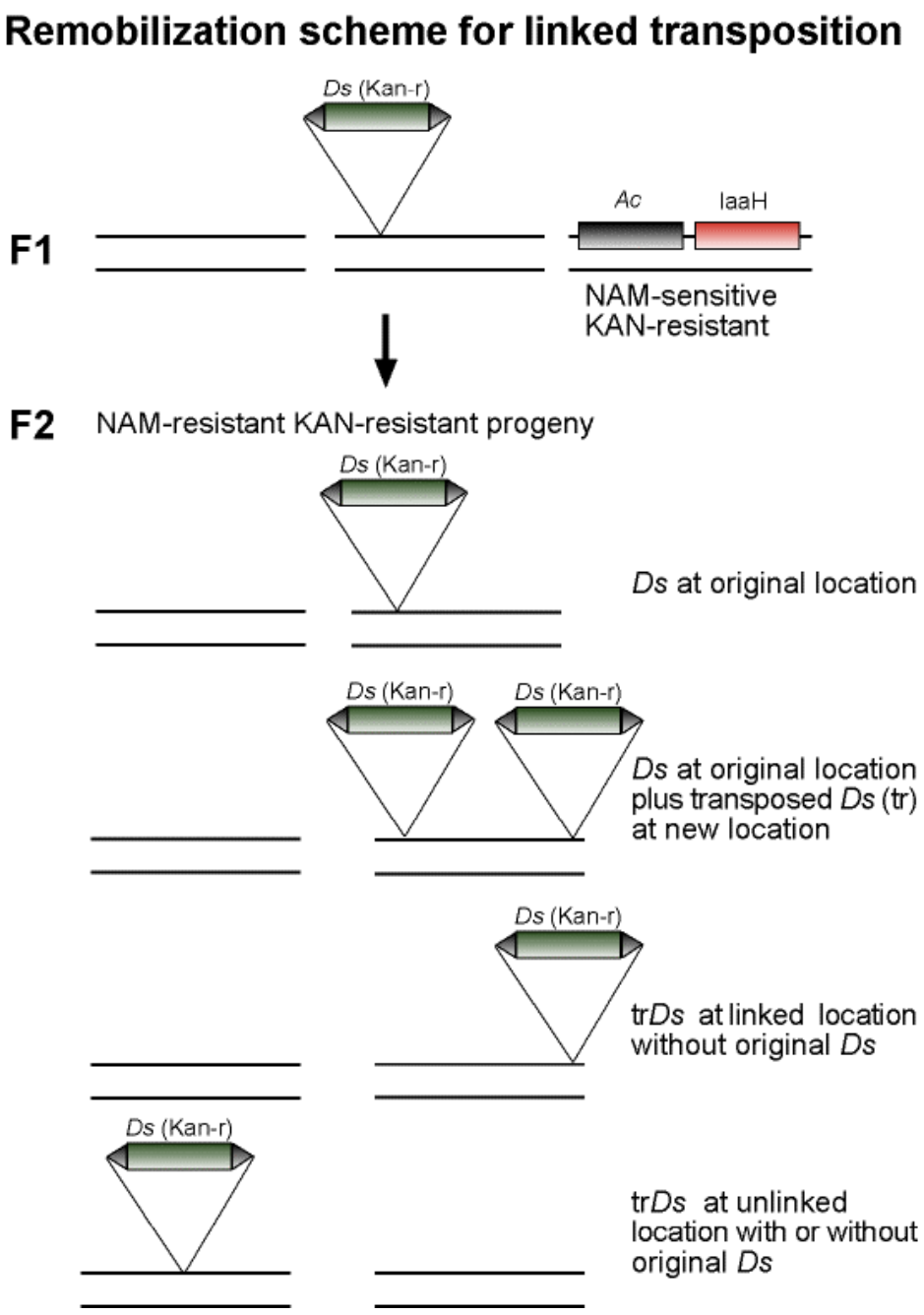

Figure 5. Re-mobilization scheme for linked transpositions. Mutagenesis crosses and the expected classes of mutagenenized progeny are shown as described in Protocol 10. In this case the Ds element at the original locus is not flanked by the IAAH gene, so that Ds at the original locus and linked transpositions are not selected against. They will therefore predominate among the F2 progeny. F2 plants carrying the Ac transposase gene are selected against in order to stabilize transposed elements. NAM, naphthalene acetamide; IAAH indole acetamide hydrolase; kan, kanamycin A.

Protocol 1A. Growth of Arabidopsis

Equipment and Reagents

- Greenhouse or plant growth cabinets
- Arabidopsis soil mix JI#A: 150 l Intercept® (Levingtons) compost with 54.4 l fine grit (small volume) or 600 l Intercept® compost with 217.6 l fine grit (large volume). Transfer the compost to 60 cell trays from PlantPac (horticultural suppliers) and firm in gently and top up. Immediately before use water trays then drench with Nemasys® (nematode) (BioCentre).
- 0.15% Agar: 150 mg Micro Agar (Duchfuca) in 1 l dH_20, autoclave sterilized.
- 15ml or 50 ml Falcon tubes
- Multi-dispenser (100µl aliquots from 6ml)

High through-put sowing, plant growth and harvesting

1. Weigh Arabidopsis seed to determine required seed number (100/2.5 mg) and place in a Falcon tube of the required volume.
2. Calculate volume of agar solution require to give 1 seed/100µl eg. 60 seed requires 6 ml.
3. Add the required volume of 0.15% agar solution to the falcon tube containing the seed and mix by inversion.
4. Chill the seed/agar mix at 4°C for 4 days to break dormancy.
5. Cut the tip from a multi-dispenser tip to prevent blockage when dispensing the seed/ agar mix. Re-shake the tube to mix the seed evenly. Draw up the seed/agar mix into the multi-dispenser. Set the dispense volume to 100 µl and dispense 100 µl aliquots into each cell of the seed tray.
6. Cover the trays with horticulture fleece; recheck for watering each day. Start to lift the fleece for short periods to allow air circulation at day 3.
7. At day 7 thin/prick out seedlings covering with the fleece for 2 days during weaning.
8. At day 10 tube the plants and start bottom watering.
9. Plants will generally have completed flowering after 3 months. Start to reduce the watering after 2.5 months to stimulate seed set. Stop watering when approximately 50% of the sliques are brown. Fully dry the plants before harvest.

Seed processing/threshing

10. Ensure the seed have fully dried before threshing.
11. Run your hand over the seed bag against a bench to open the sliques (brushing).
12. Shake the seed into one corner of the bag.
13. Place two tea strainers, one inside the other over the mouth of a new seed bag. Cut off the corner of the seed bag containing the seed and empty the seed through the tea strainer into the new seed bag (threshing). This will remove sufficient chaff to provide seed suitable for sterilization prior to *in-vitro* work.

Protocol 1B. Mutagenesis

Equipment and Reagents

- Fine pointed forceps
- Dissecting microscope with fiber optic light source
- Greenhouse or plant growth cabinets and soil

Generation of F1 and F2 seed

1. Sow parental seed homozygous for the transposase gene in individual 2" pots and grow under optimal conditions: 16 hour days are recommended. Plant homozygous *Ds* seed one week later.
2. Emasculate the five buds from each *Ds* parent (before pollen is shed) and remove the remaining buds. Up to 60 plants may be prepared in a day. Leave each flower overnight before pollination with homozygous transposase pollen. Cover the fertilized buds with a small square of cling film to prevent loss of seed upon silique shattering. Remove any unfertilized buds, and mark the branch. Over the next 2 weeks remove new flowers and branches.
3. Collect the seed approximately 2 weeks later, just before the siliques open.
4. Plant the F1 seed individually with a damp spatula on moistened compost in the greenhouse. On bolting, provide each plant with a small plastic or wire stake, and a 12" polythene collection tube to avoid seed contamination.
5. Harvest 5-10,000 F2 seed from each F1 plant. It is convenient to work in batches of 2-4,000 F1 plants at a time. Avoid seed contamination.

Starter lines

Rob Martienssen's lab have provided four *DsG* starter lines and four *Ac* starter lines:

*DsG*1 (3734-1)	*Ac*1 (4345-1)
*DsG*6 (4224-1)	*Ac*2 (4562-9)
*DsG*7 (1536-9)	*Ac*4 (3680-2)
*DsG*8 (4225-1)	*Ac*5 (4347-4)

For optimal results these should be crossed in the following combinations:

1. *DsG*1 (3734-1) X *Ac*4 (3680-2)
2. *DsG*6 (4224-1) X *Ac*4 (3680-2)
3. *DsG*6 (4224-1) X *Ac*1 (4345-1)
4. *DsG*7 (1536-9) X *Ac*2 (4562-9)
5. *DsG*8 (4225-1) X *Ac*4 (3680-2)
6. *DsG*8 (4225-1) X *Ac*5 (4347-4)

Protocol 2: Selecting transposants from F2 families

Equipment and Reagents

- Germination media: 4.2 g/1 MS salts (GIBCO/BRL), 10.0g/1 glucose (tissue culture grade, Sigma), pH to 5.7 with 1 M KOH.
- Plates: Add 7 g agar per liter GM and autoclave. Allow to cool to 60°C, then add 325 µ 1 per litre NAM (alpha-napthalene acetamide, Aldrich 127752) from a 2 mg/ml stock in 95% EtOH. Add 1 ml kanamycin A (Sigma K-4000) from a 50 mg/ml filter sterilized stock. (Final concentrations are 0.65 µ g/ml NAM, 50 µg/ml kanamycin A). Pour plates at least 8 mm deep; this is important for the selection.
- Top agar: Add 7 g agar per liter GM and autoclave. This can be stored at room temperature, and melted in the microwave before using. Cool to 60°C then add 325 µ 1 per litre NAM (alpha-napthalene acetamide, Sigma) from a 2 mg/ml stock in 95% EtOH and add 1 ml kanamycin A from a 50 mg/ml filter sterilized stock before use.
- 95% Ethanol.

Selection of F2 transposants.

1. Aliquot F2 seed into approximately 700 seed (15 mg) batches in 15 ml plastic tubes.
2. Sterilize the seed by following the protocol below. Work in the hood, and use sterile technique.

- Wash with 2ml bleach solution, (50% bleach / sterile water, 0.05% Tween 20, vortex individual tubes and leave for no 5 minutes, no longer. Tip off.
- Wash with 2ml 70% Ethanol, vortex and leave for 5 minutes, no longer. Tip off.
- Fill tube with sterile water. Allow seed to settle, and decant off.

3. Add top agar to 6 ml using Duran top fitting dispenser (Labmax dispenser, Alpha Labs). Swirl gently to evenly distribute seeds.
4. Place at 4°C for 4 days to encourage even germination (in darkness), and then move plates to a growth chamber at 21°C for 4-5 days (initial growth in the dark improves selection). Unwrap the plates and grow at 21°C under reduced light for 1-2 days (use Osram daylight lamps L58860, 58W T8 or equivalent daylight colour 54 lamps from GE or Sylvania), (the reduced light encourages hypocotyl elongation, which enhances NAM selection).
5. Seedlings resistant to NAM can be recognized by their long, unbranched roots, long hypocotyl and upright stature. Most of these will be pale green indicating kanamycin sensitivity. Select those NAM resistant seedlings that are dark green (kanamycin resistant) and transfer onto fresh NAM/KAN GM plates. Use sterile forceps, and work in the hood. After 5-7 days, the phenotypes will be more extreme on fresh rather than on the crowded plates, and easily recognized. Always transfer some sensitive seedlings as a control.
6. Transfer seedlings to soil when the phenotypes are unambiguous. The number of double resistant seedlings varies from 0 to 2.5% in each family and the proportion of families transposants is approximately 20%. Transplant from 1-5 seedlings to soil, but thin all but one plant on flowering. Collect F3 seed.

Protocol 3: Screening F3 seedlings for reporter gene expression

Equipment and Reagents

GUS stain:

FERICYANATE STOCK: 100 mM	10 ml
$K_4Fe(CN)_6.3H_2O$	422 mg
$K_3Fe(CN)_6$	329 mg
$3H_2O$	10 ml

PHOSPHATE BUFFER pH7.0: 100 mM	1l

Na_2HPO_4: 1 M
NaH_2PO_4: 1 M
57.7 ml Na_2HPO_4: 1 M + 42.3 ml NaH_2PO_4: 1 M dilute to 1 l in dH_2O

STAINING BUFFER	50 ml
X-Gluc	25 mg in 500 µl DMF
Triton X100: 0.1%	50 µl
Fericyanide: 0.5 mM	250 µl 100 mM stock
Phosphate buffer pH7.0: 50 mM	25 ml 100 mM stock
dH_2O	to 50 ml

store at -20°C

HEPTANE
recycle after use.

FIXATIVE	25 ml
glutaraldehyde 1%	1 ml (25%)
formaldehyde 4%	2.7 ml (37%)

CHLORAL HYDRATE CLEARING BUFFER

	100 ml
Chloral hydrate	67 g
dH_2O	25 ml
glycerol	8.3 ml

Plating and Sterilization

1. Sterilize 20 – 30 seeds from each F3 in an eppendorf tube according to protocol 2, but using smaller volumes.
2. Resuspend seed in 0.7 ml 0.1% agar. Spread on 60 mm GM plates without selection, distributing evenly.
3. Place plates at 4°C for 4 days, then transfer plates to growth chamber for 4 – 8 days.

Staining procedure

1. Dissect out tissues for staining. Place tissue in a multi-compartment petri dish. Add 1 – 2 ml 100% heptane for 10 min. Remove heptane (& recycle); air dry for 5 min.
2. Add 1 ml of X-Gluc solution. Place in a vacuum dessicator and draw vacuum for 10 min, then incubate at 37°C. Run a time course for staining at 30 min intervals for 5 h and o/n. Remove the staining solution.
3. Post staining fixation: add 1 – 2 ml of 1% glutaraldehyde / 4% formaldehyde. Place in a vacuum dessicator and draw vacuum for 10 min then fix o/n at 4°C. Tissue may now be stored for embedding or cleared for whole mounts.
4. Clear in 95% ethanol for 1 h then 70% ethanol for 2 h for routine screening. For more detailed analysis clear with chloral hydrate solution for 1 h. Replace the clearing solution with fresh chloral hydrate solution, mount on a slide in the clearing solution. Apply a cover slip and view using Nomarski optics.

Protocol 4A: Amplification of flanking DNA
Inverse PCR

Genomic DNA flanking the insertion site can be readily amplified by a number of PCR procedures. The most commonly used methods are IPCR and TAIL PCR.

Inverse PCR

Inverse PCR is performed on genomic DNA. The DNA is digested with an appropriate restriction enzyme then ligating under dilute conditions to generate circular fragments. Finally the insertion site is amplified by PCR. This protocol is designed to conduct multiple IPCR reactions in a 96 well microtitre plate.

Equipment and Reagents

1. Digestion:

8.8µl (50ng) DNA, 1µl 10 X BstYI buffer (NEB), 0.1µl BstYI (10U/µl), 0.1µl 100 X BSA (10mg/ml). Digest at 60°C for 1 hour and 30 minutes. Inactivate the enzyme at 75°C for 20 minutes.

For 96 reactions:
102µl 10 X BstYI buffer (NEB),
10.2µl BstYI (10U/µl),
10.2µl 100 X BSA (10mg/ml)
Aliquot 1.2µl / sample
Add 8.8µl DNA per sample
Run **DigestB**

2. Ligation:

10µl digest, 4µl 10 X ligase buffer (BRL), 0.03µl T4 ligase (5U/µl), 25.97µl dH_2O. Ligate overnight at 4°C in a refrigerator.

For 96 reactions:
408µl 10 X ligase buffer (BRL)
3.06µl T4 ligase (5U/µl Roche)
2648.94µl dH_2O
Add 30µl / sample
Ligate overnight at 4°C in a refrigerator.

3. Primary PCR

PCR reaction mix:

4µl DNA (from ligation), 2µl 10 X PCR, 2.5µl dNTPs (2 mM), 0.6µl primer A (10µM), 0.6µl primer B (10µM), 0.2µl Tag/Pwo (5U/µl) (Ratio Taq/Pwo [Boehringer] of 160U/1U), 10.1µl dH_2O
(10 X PCR buffer: 17.5 mM $MgCl_2$, 500 mM Tris-Cl pH 9.2, 140 mM $(NH_4)_2SO_4$)

For 96 reactions:
204µl 10 X PCR
204µl dNTPs (2.5 mM)
30.6µl primer A1 (20µM)
30.6µl primer B (20µM)
20.4µl Tag/Pwo (5U/µl) (Ratio Taq/Pwo [Boehringer] of 160U/1U)
1142.4µl dH_2O
Aliquot 4µl / sample
Add 16µl reaction mix per sample
Run **3-IPCR** or **5-IPCR**

4. Nested PCR
PCR reaction mix:
1µl DNA (from 1 in 50 dilution of primary reaction)**, 4µl 10 X PCR, 4µl dNTPs (2.5mM), 0.6µl primer A (20µM), 0.6µl primer B (20µM), 0.4µl Tag/Pwo (5U/µl) (Ratio Taq/Pwo [Roche] of 160U/1U), 29.4µl dH_2O
For 96 reactions
408µl 10 X PCR
408µl dNTPs (2.5mM)
61.2µl primer A2 (20µM)
61.2µl primer B (20µM)
40.8µl Tag/Pwo (5U/µl) (Ratio Taq/Pwo [Roche] of 160U/1U)
2998.8µl dH_2O
Aliquot 1µl diluted primary PCR / sample
Add 16µl reaction mix per sample
Run **3-IPCR** or **5-IPCR**

5. PCR conditions:
3-IPCR
94°C 2 min 1cycle; 94°C 15 sec, 65°C 30 sec, 68°C 5 min 38 cycles; 68°C 5 min 1 cycle.
5-IPCR
94°C 2 min 1cycle; 94°C 15 sec, 55°C 30 sec, 68°C 5 min 38 cycles; 68°C 5 min 1 cycle.
IPCR primers:
Primer A:
[A1] 5' *Ds* : **B34** 5' ACG GTC GGT ACG GGA TTT TCC CAT 3'
[A2] **4447** 5' CCG TTT CCG TTC CGT TTT C 3'
[A1] 3' *Ds* : **340** 5' CTT ATA TGG CTT CTT ATG TTA GCC AAG AG 3'
[A2] **B39** 5' TTC GTT TCC GTC CCG CAA GTT AAA TA 3'
Primer B
5' *Ds* : **B35** 5' TAT CGT ATA ACC GAT TTT GTT AGT TTT ATC 3'
3' *Ds* : **DL6** 5' TTG CAG CAG CAA TAA CAG AGT CTA GC 3'

6. 1.2% Gel:

   Run 8µl of 3° amplification products on a 1.2% gel to check for fragments. If the reaction is successful there will be 50-100ng of product in each lane. Multiple products should be anchored by the Ds primer and should not confuse sequencing reactions.

7. Product Purification:

   Purify the remaining 22µl of successful 3° products by running over a Qiaquick PCR purification kit to remove primers and nucleotides.

   Add 100µl of PB buffer (5x vol) and mix
   Load onto columns and spin
   Wash 2 x with 900µl PE and spin
   Place columns onto collection tubes and elute with 60µl EB
   Stand for 1 minute then spin

8. Sequencing:

   Sequencing directly using 5-10µl (8µl) of the 3° product as template using dye terminator chemistry and primer B39 or 4447.

NOTES

** Always spin plates after mixing dilutions

Protocol 4B: Amplification of flanking DNA
TAIL PCR

A. Amplification of Flanking DNA:

Genomic DNA flanking the insertion site can be readily amplified by a number of PCR protocols. The most widely used method in the Marteinssen lab is Tail PCR.

**Use PCR dedicated pipetman to make up all PC solutions.
**Use filter-tips for all steps.
**Use Analar water for consistency
**TAIL PCR IS EXTREMELY SENSITIVE TO PCR CONTAMINATION.

B. Reagents:

- Primer solutions: (final concentrations)

DS3/Ds5-1N – Ds3/Ds5-4N (20µM)
AD2 primer (20µM)
10x PCR buffer (15mM Mg^{+2})
dNTP's (2mM)
Taq Polymerase (5 units/µl)
dNTP's: 100mM stock to 2mM working stock
20µl of each A,C,G,T + 920µl dH_2O (1000µl)

C. Primers:

[1] Adapter Primer:

AD2: 5'-NGT CGA (G/C)(A/T)G ANA (A/T)GA A-3'

[2] *Ds* Specific Primers: - 3' *Ds*

Ds3-1: 5'-ACC CGA CCG GAT CGT ATC GGT-3'
Ds3-2: 5'-CGA TTA CCG TAT TTA TCC CGT TC-3'
Ds3-4: 5'-CCG TCC CGC AAG TTA AAT ATG-3'

[3] *Ds* Specific Primers: -5' *Ds*

Ds5-1: 5'-ACG GTC GGG AAA CTA GCT CTA C-3'
Ds5-2: 5'-CCG TTT TGT ATA TCC CGT TTC CGT-3'
Ds5-5: 5'-CGAAATCGATCGGGATAAA-3'

D. Protocol:

[**1**] 1° Amplification: (20µl volume reaction)

	1 x Reaction	**96 Reactions**
Ds3-1N **OR** Ds5-1N (20µM)	0.2µl	20µl
AD2 (20µM)	3µl	300µl
10x PCR Buffer (15mM Mg^{+2})	2µl	200µl
dNTP's (2mM)	2µl	200µl
dH_2O (Analar)	12.6µl	1260µl
Taq polymerase	0.2µl	20µl

Aliquot 20µl per sample
Add 1µl DNA per sample
Run **Tail 1**

[**2**] 2° Amplification: (20µl total volume reaction)

	1 x Reaction	**96 Reactions**
Ds3-2N **OR** Ds5-2N (20µM)	0.2µl	20µl
AD2 (20µM)	3µl	300µl
10x PCR Buffer (15mM Mg^{+2})	2µl	200µl
dNTP's (2mM)	2µl	200µl
dH_2O (Analar)	12.6µl	1260µl
Taq polymerase	0.2µl	20µl

Aliquot 20µl per sample
Add 1µl DNA of a 1:50 dilution of 1° amplification (1µl DNA + 49µl dH_2O).
Run **Tail2**

[**3**] 3° Amplification: (30µl total volume reaction)

	1 x Reaction	**96 reactions**
Ds3-4N **OR** Ds5-5N (20µM)	0.3µl	30µl
AD2 (20µM)	3µl	300µl
10x PCR Buffer (15mM Mg^{+2})	3µl	300µl
dNTP's (2mM)	3µl	300µl
dH_2O (Analar)	20.4µl	2040µl
Taq polymerase	0.3µl	30µl

Aliquot 30µl per sample.
Add 1.5µl DNA of a 1:10 dilution of 2° amplification (1.5µl DNA + 13.5µl dH_2O).
Run **Tail3**

A. Notes:

* Prepare 96 reaction stocks in advance excluding Taq polymerase and store at -20°C. Before use add the Taq, mix and use the MATRIX boats for dispensing.

[**4**] **1.2% Gel**:

Run 8µl of 3° amplification products on a 1.2% gel to check for fragments. If the reaction is successful there will be 50-100ng of product in each lane. Multiple products should be anchored by the Ds primer and should not confuse sequencing reactions.

[**5**] **Product Purification**:

1. Purify the products using the following method.
2. Put 5µl of PCR product into the PCR tubes used for sequencing.
3. Add 1µl of Shrimp Alkaline Phosphatase and 1µl of Exonuclease I.
4. Incubate using PCR program **CLEANUP.**

[**6**] **Sequencing:**

1. To the purified product add 5.5µl of **Terminator and primer mix.**
2. Run on PCR program **Term**.
3. After sequencing reaction add 60µl 95% Ethanol and 1µl 250mM EDTA to each well
4. Incubate on ice for 30 minutes.
5. Spin at 3000rpm for 30 minutes.
6. Gently tip out liquid and add 100µl 70% ethanol
7. Spin at 3000rpm for 10 minutes
8. Spin inverted on a piece of blue roll at 200 – 300 rpm for 5 minutes.
9. Allow to air dry and sequence.

Terminator and primer mix

	1	**48**	**96**
ABI Terminator Mix	4µl	202µl	403µl
Ds3-4N/Ds5-5N primer (20µM)	0.2µl	5.8µl	11.6µl
Water	1.3µl	70µl	140µl

NOTES

** Always spin plates after mixing dilutions

E. PCR Programmes

Tail1 Run time 4 hr 16 minutes

94°C for 1 minute
94°C for 10 seconds
62°C for 1 minute
72°C for 2:30 minutes
Goto step 2, four times
94°C for 10 seconds
25°C for 3 minutes
ramp 0.2°C/s to 72°C
72°C for 2:30 minutes
94°C for 10 seconds
68°C for 1 minute
72°C for 2:30 minutes
94°C for 10 seconds
68°C for 1 minute
72°C for 2:30 minutes
94°C for 10 seconds
44°C for 1 minute
72°C for 2:30 minutes
Goto step 10, fourteen times
72°C for 5 minutes
4°C forever
End

Tail2 Run time 3 hr

94°C for 10 seconds
64°C for 1 minute
72°C for 2:30 minutes
94°C for 10 seconds
64°C for 1 minutes
72°C for 2:30 minutes
94°C for 10 seconds
44°C for 1 minute
72°C for 2:30 minutes
Goto step 1, eleven times
72°C for 5 minutes
4°C forever
End

Tail3 Run time 1 hr 52 minutes

94°C for 15 seconds
44°C for 1 minute
72°C for 2:30 minutes
Goto step 1, nineteen times
72°C for 5 minutes
4°C forever
End

CLEANUP

37°C for 30 mins
80°C for 10 mins
4°C Forever
End

Term

1.0°C/s to 96°C
96°C for 2 minutes
96°C for 10 seconds
1.0°C/s to 50°C
50°C for 5 seconds
1.0°C/s to 60°C
60°C for 4 minutes
goto step 3 twenty four times
4°C forever
End

Protocol 5: Amplification of gene trap cDNA by 5' RACE PCR

Equipment and Reagents

- Purified total poly(A)+ RNA isolated from line of interest;

- GUS specific primers;

GUS2: 5'-TTT TCG GGA TCC AGA CTG AAT GCC CAC AG- 3'
GUS3: 5'-GAG AAT TCT TGT AAC GCG CTT TCC CAC CA- 3'
GUS4: 5'-GCT CTA GAT CGG CGA ACT GAT CGT TAA AAC- 3'

- RACE primers

QO: 5'-CCA GTG AGC AGA GTG ACG- 3'
QI: 5'-GAG GAC TCG AGC TCA AGC- 3'
QT: 5'-CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GCT TTT TTT TTT TTT TTT- 3'

- RNase inhibitor (Roche)
- AMV Reverse Transcriptase (Roche)
- 5X Reverse Transcriptase buffer (250 mM Tris, pH8; 30mM $MgCl_2$; 200mM KCl; 5mM DTT; 5mM each dCTP, dATP, dGTP, dTTP)
- Terminal Deoxynucleotidyl Transferase (TdT) (Gibco BRL)
- Taq polymerase
- Centricon 100 spin columns (Amicon Corp., Danvers, MA)
- PCR machine

cDNA synthesis and homopolymer tailing

1st strand synthesis

1. Perform first strand cDNA synthesis using 5 – 10 µg total RNA or 1µg Poly(A)+ RNA and 10 pmol GUS4 primer. A reaction in which the reverse transcriptase is omitted is a used as a control. Incubate at 70°C for 5 min; chill on ice. Add 4 µl 5X buffer, 1 µl GUS4 primer. 0.5 µl Rnase inhibitor and water to 19.5 µl. Add 0.5 µl (20U) AMV Reverse Transcriptase. Incubate at 42°C for 1 h, followed by 52°C for 30 min.

2. Dilute to 2 ml with 1X TE and concentrate with Centricon 100 spin column. Wash with 2 ml 0.2X TE. This removes excess primer. Concentrate in SpeedVac to 10 µl. Add 4 µl 5X TdT buffer, 4 µl 1 mM dATP and 1 µl TdT. Incubate 5 min at 37°C followed by 5 min at 65°C to stop the reaction.

3. Ethanol precipitate the tailed cDNA using tRNA as a carrier and resuspend in 20 µl dH_2O. Store at –20°C.

2nd strand synthesis

1. Set up first round amplification reactions using 1 µl of cDNA, 25 pmol each of primers QO and GUS3 and 2pmol of QT to 50 µl of PCR cocktail [1x *Taq* polymerase, dNTPs at 1.5 mM, 10% DMSO]. Heat to 97°C then cool to 75°C. Add 2.5 U of *Taq* polymerase and incubate at 48-52°C for 2 min. Extend the cDNAs at 72°C for 40 min. Carry out 30 cycles of amplification (94°C, 1min; 48-52°C, 1 min; 72°C, 3 min), followed by a final extension for 15 min at 72°C. Cool to room temperature. Controls should include reactions using single primers.
2. Dilute products from the first round 20 fold and amplify 1 µl of diluted products with QI and GUS2 primers using the procedure described in step 1, but eliminating the initial 2 min annealing step and the 72°C, 40 min extension step.
3. Run products from both first and second round amplifications on a gel to analyze. Gel blot hybridization can be used to confirm the authenticity of the products.

Products are purified using QiaQuick PCR purification columns (Qiagen). They can be cloned into appropriate vectors by digesting with *Bam* HI (site present in GUS2) and *Hin* dIII (present in QI).

Protocol 6: Remobilization of transposed elements

1. Sow parental seed homozygous for the transposase gene in individual 2" pots and grow under optimal conditions: 16 hour days are recommended. Seed from plants homozygous for the insertion one week later.
2. Emasculate the five buds from each *Ds* parent (before pollen is shed) and remove the remaining buds. Leave each flower overnight before pollination with homozygous transposase pollen. Cover the fertilized buds with a small square of cling film to prevent loss of seed upon silique shattering. Remove any unfertilized buds, and mark the branch. Over the next 2 weeks remove new flowers and branches.
3. Collect the seed approximately 2 weeks later, just before the siliques open.
4. Plant the F1 seed in batches of 50-100 each in 50 large pots. Harvest F2 seed pot by pot.
5. Select F2 seed on NAM/KAN plates (see Protocol 2). Use 3-4 plates per pot (150-200 plates).
6. Identify 2,000 double resistant seedlings from 50 pools and transplant to soil. Plant in the greenhouse in a 2-dimentional grid of 256 small pots (16 rows x 16 columns), planting 8 plants per pot.
7. On flowering, collect flowers from all plants in each row and column of the grid: ie. Collect 128 inflorescences from each row, and 128 from each column.
8. Prepare DNA from the pools of flower tissue (32 DNA preps total).
9. Harvest seed from each pot as a pool (8 plants each).
10. Amplify DNA using the primers from the target gene and primers for the Ds element. Perform separate amplifications using Ds3-1 (5'-ACC CGA CCG GAT CGT ATC GGT-3') and Ds5-1 (5'-ACG GTC GGG AAA CTA GCT CTA C-3')

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