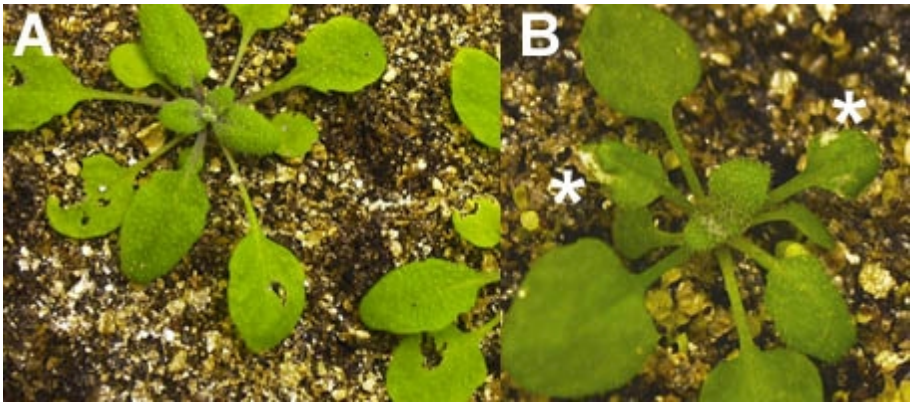
PhD Project Protocols

Following are the protocols used for this project. The transformation method of Bechtold has been improved over the original method and these modifications are available in publications.

Pest control

Most of the problems we have had so far are with thrips and flies (along with their larvae). The small black fly like insects are difficult to get rid of and lay eggs in the soil. Their worm-like larvae usually feed off organic matter in the soil before hatching. They also sometimes have a hankering for young Arabidopsis. They usually eat out the roots and also eat leaves that are touching the ground (A, below). They make holes in the leaves and the leaves sometimes respond by producing anthocyanins (going purple). The larvae look like worms, are clear in colour, varying in size as they get older and at their oldest are up to 5mm long (much bigger than the fly). Features that make them unlike worms are the fact that they eat plants, have mandibles and a solid head (not soft and contracting like earthworms). We use sticky fly strips to keep adult numbers down as much as possible and when we have outbreaks we control them with Diazanon again at the same concentration for thrips. They don't seem to bother older plants, presumably they are too tough to eat.



The best way to control flies is to implement a biological control program. We received monthly deliveries of nematodes which are applied to the soil and feed on the larvae of black fly but are harmless to Arabidopsis plants. The nematode is sold in Australia as Gnatem by Ecogrow (Offshoot company from CSIRO Entomology) but presumably other countries have the similar resources available. Contact (from other countries)+61-2-93890888 or (within Australia) (02)93890888.

We have also experienced some problems with thrips. Thrips are sap-sucking insects that get really stuck into Arabidopsis. If left unchecked they can kill most of the plants in your room. They are more damaging to young plants than to old. They are really small (can just be seen with the naked eye) and depending on their age have wings. They have very resistant egg stage which are always laid on the attacked plant. Eggs can actually be laid so they become within the seeds of an infected plant. The marks that they make on the plants are usually white/clear lines on the leaf where they have sucked the contents out of the cells (B above). The leaves can wrinkle in response to attack. To control we use a systemic insecticide called Diazanon at the lowest recommended concentration used (instructions suggest this concentration for bean fly). Too much will damage the plants.

Although Rogor is recommended to control thrips we found this very rough on Arabidopsis.

DNA manipulation

Alkaline lysis [3] was used for all plasmid DNA extractions from bacteria. E.coli (strain DH10B) grown on LB media was used for all cloning steps and was transformed by electroporation. High purity binary vector DNA for cloning and sequencing was prepared by standard alkaline lysis as described by Jones et al. [1] with modifications. E.coli were pelleted by centrifugation at 13,000 ug for 10 min and then incubated for 5 min with lysozyme (400 ug/ml) in 1 ml glucose:Tris:EDTA per 50 ml of culture. Cells were lysed in NaOH/SDS and neutralized rapidly with potassium acetate/acetic acid (see Sambrook et al., 1989 for standard alkaline lysis solutions). The final mixture was centrifuged at 30,000 xg for 15 min to pellet cellular debris and transferred to a new tube. Cellular debris was frequently carried over so centrifugation was usually repeated. The clear supernatant was precipitated with 0.7 volumes of isopropanol, centrifuged at 4,200 xg, drained and the air-dried pellet dissolved in 1 ml TE buffer. This solution was brought to 2 M LiCl and incubated on ice for 20 min. High molecular weight RNA was pelleted by centrifugation at 4,200 xg for 15 min. The supernatant was extracted twice with an equal volume of 25:24:1 phenol: chlorofom: isoamyl alcohol and precipitated with 2 volumes of ethanol (no salt). DNA was pelleted by centrifugation at 4,200 xg at 4ºC for 20 min. The pellet was washed, air dried and dissolved in 300 ul of TE containing 20 ug of RNAase. This digestion was extracted once with phenol:chlorofom:isoamyl alcohol to remove the RNAase, brought to 0.3 M sodium acetate and DNA precipitated with 2 volumes ethanol. The mix was incubated at –20ºC for 10 min and spun at 14,000 xg for 15 min. The pellet was washed, air dried and dissolved in 50 ul of TE per 100 ml of initial culture volume. Typical yields were 0.15-0.35 ug DNA per ml of starting culture.

Blunt ending of 5’ or 3’ overhangs was performed by incubating DNA in a 100 ul reaction consisting of 50 mM Tris (pH 7.5), 10 mM MgCl2 1 mM DTT, 0.05 ug/ul BSA, 20 uM dNTPs, 1 mM ATP and 10 units each of T4 polynucleotide kinase (PNK) and Klenow polymerase.

Ligations

Binary vectors were digested by standard laboratory methods [2]. DNA was digested for 3 hours with approximately 4 units of enzyme per microgram of DNA. DNA was recovered by precipitation with 2.0 vol. EtOH (no salt), immediate centrifugation, washing with 70% EtOH and resuspension in TE buffer. All steps were performed at room temperature. Large amounts of pre-cut vector with different enzyme combinations were prepared. To reduce re-ligation of incompletely cut sticky end plasmids, digested plasmid was treated with 4 units of calf intestinal alkaline phosphatase (CIAP) at 50ºC for 1 hour and recovered as described above. Each sticky-blunt ligation mix contained 400-500 ng of plasmid and 500-1000 ng of insert DNA (depending on size). Each sticky-sticky ligation mix contained 200-300 ng of plasmid and 250-500 ng of insert DNA.

1. Jones JD, Shlumukov L, Carland F, English J, Scofield SR, Bishop GJ, Harrison K: Effective vectors for transformation, expression of heterologous genes, and assaying transposon excision in transgenic plants. Transgenic Research 1: 285-297 (1992).

2. PGEL: Plant Genetic Engineering Laboratory: Basic Molecular Biology Techniques and Lab Procedures. www.uq.edu.au/~uqpgel, University of Queensland, Brisbane (2000).

3. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, New York (1989).

Agrobacterium / Transformation Tri-Parental Mating

The method used for tri-parental mating was based on that used by Svab et al. [2]. E.coli containing the appropriate binary vector was streaked onto LB-Tet10. A loop of (1.) TetR E.coli with binary vector, (2.) KmR helper E.coli HB101/pRK2013 and, (3.) RifR Agrobacterium tumefaciens (strain LBA4404 or GV3101) was mixed on an LB plate and incubated at 28ºC for 20 hours before streaking onto LB-Tet2/Rif50 to select for Agrobacterium containing the TetR binary plasmid. An individual colony was re-streaked onto LB-Tet2/Rif50 before performing colony PCR with 35S and nos primers to confirm the presence of the correct binary construct.

In planta transformation

In preparation for the transformation, Agrobacterium was streaked onto triple selection LB media to ensure the presence of the virulence plasmid (Tet2/Rif50/Strep25 for LBA4404, Tet2/Rif50/Gen25 for GV3101) before growth in LB-Tet2/Rif50 broth. Arabidopsis plants used for vacuum infiltration were grown at 21ºC under 50 uE/m2/sec of long day light (16:8 light/dark) provided by white fluorescent tubes. As plants bolted, Agrobacterium cultures were prepared for in planta transformation as described by Bechtold et al. [1]. After infiltration, pots were placed in humid containers under lights overnight, uncovered and allowed to grow to full maturity before seed was harvested. For each construct, in planta transformation was performed in five 8 cm pots containing 5-15 plants per pot.

Basta Selection

Selection of transgenics was performed by sowing approximately 0.5 ml of seeds from infiltrated plants in soil trays (260 mm x 310 mm). Germination was synchronised by treatment at 4ºC for 3-5 days. Trays were placed under long-day light at 21ºC and seedlings sprayed first upon emergence and twice afterwards at 3-day intervals with 0.4% Basta (Basta®, active constituent 20% glufosinate ammonium; Hoechst Schering Agrevo GmbH). Basta-resistant plants were transferred to pots and grown to maturity. Plants were observed during growth for the presence of visible phenotypes.

Plant Growth for Segregation Analysis

100-200 T2 seeds were sown in a non-sterile petrie dish on a filter paper moistened by 8 g ground vermiculite soaked in approximately 25 ml of 0.0066% Basta. Germination was synchronised at 4ºC for 3 days and then plates were placed under 24 hr light for 7 days and scored for segregation ratio. Basta sensitive plants germinate but immediately become chlorotic. Basta resistant plants germinate and remain green but do not progress past the cotyledon stage. Click here for more detail.

1. Bechtold N, Ellis J, Pelletier G: In planta Agrobacterium mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. Comptes Rendus de l'Academie des Sciences Serie III Sciences de la Vie 316: 1194-1199 (1993).

2. Svab Z, Hajdukiewicz P, Maliga P: Generation of transgenic tobacco plants by cocultivation of leaf disks with Agrobacterium pPZP binary vectors. In: Maliga P, Klessig DF, Cashmore AR, Gruissem W, Varner JE (eds) Methods in plant molecular biology : a laboratory course manual, pp. 55-77. Cold Spring Harbor Laboratory Press, Plainview, NY (1995).

Transgenic Analysis

Phenotype co-segregation with Basta resistance

Co-segregation of mutant phenotypes with Basta resistance was determined by painting leaves of three-week old T2 plants with 0.08% Basta (Basta®, active constituent 20% glufosinate ammonium, Hoechst Schering Agrevo GmbH) and scoring their response to herbicide two days later. Late appearing phenotypes may be scored for Basta resistance and examined for the phenotype later.

Southern analysis

Genomic DNA was recovered from the LiCl supernatant of RNA extractions (see 'northern analysis' below) by ethanol precipitation, washed and resuspended in T.E. 3 ug of genomic DNA was digested overnight with EcoRI, HindIII or SalI, fractionated on a 0.8% agarose (0.5X TBE) gel and the blot prepared by standard methods [4]. For the broomhead mutants, only 1.2 ug of DNA was digested. Probes were hybridized to the blots overnight in Church buffer [1] and washed in 2 x SSC, 0.1% SDS at room temperature for 15 min; 2 x SSC, 0.1% SDS at 55 ºC for 15 min and twice in 0.2 x SSC, 0.1% SDS at 55 ºC for 10 min. The 35S probe was amplified in a standard 35 cycle PCR reaction using T7 and SP6 primers in combination with pZLD-35S as template. The reaction was extracted with 1 volume of 25 : 24 : 1 of phenol : chloroform : isoamyl alcohol and recovered by ethanol precipitation with sodium acetate as previously described. Individual EST probes were also produced in this manner but using the original PRL-2 plasmid preparation as template. Probes were radio-labeled with 32P-dCTP by the random priming method.

Northern analysis

Total RNA from leaf tissue was extracted as described by Etheridge et al. [2], fractionated on a 1% agarose (0.5X TBE) gel and transferred to a nylon membrane by capillary blotting. Hybridization and washing conditions were the same as for Southern analysis. A probe from the wheat 25S rRNA gene [3] was used as a loading control.

1. Church GM, Gilbert W: Genomic sequencing. Proceedings of the National Academy of Sciences of the United States of America 81: 1991-1993 (1984).

2. Etheridge N, Trusov Y, Verbelen JP, Botella JR: Characterization of ATDRG1, a member of a new class of GTP-binding proteins in plants. Plant Molecular Biology 39: 1113-1126 (1999).

3. Gerlach WL, Bedbrook JR: Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Research 7: 1869-1885 (1979).

4. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, New York (1989).

Segregation analysis using Basta in non-sterile petrie dishes

To get homozygous lines usually involves tedious seed sterilization and plating but not with Basta. The following protocol details how to do segregation analysis in non-sterile petrie dishes containing 0.0066% Basta. The plants will not grow past the cotyledon stage but it is big enough to see Basta resistance vs. non-resistance. You need: 1. A non-sterile petrie dish, 2.Ground vermiculite (put vermiculaite in a blender and blend it into powder),

3.Basta (Basta is usually 200g/L glufosinate ammonium), 4.No. 1 Whatman filter paper or one you cut yourself to fit the dish

Here's what you do:

- Put about 10g of ground vermiculite on the bottom of the petrie dish and make it flat, put the filter paper on top

- Add 1 microlitre of Basta per 15 ml of water (can use 10 microlitres of 1:10 diluted Basta since full strength Basta is gluggy), pour the 15ml on top of the filter paper. The paper should go wet and the vermiculite should soak up the rest. It should be all wet but no liquid should pool on top

- Sprinkle seeds on the filter paper, it's not a bad idea to seal up the plate with micropore tape or even parafilm to prevent evaporation (which concentrates the Basta)

- Vernalize/stratify at 4 degrees as usual then put under lights and after about a week to a week and a half you should be able to distinguish easily between Basta resistant and non-resistant plants. This is only useful for segregation analysis. The plants will get sick looking and die after about 2 weeks or more. They aren't likely to make more than just the cotyledons. Non transgenics will germinate but go yellow immediately and the cotyledons don't expand much. In resistant ones the cotyledons expand and are much greener. This method will also allow you to see non germinating seed. P.S. I did try putting nutrients (0.5g/L Aquasol) in there but there wasn't much difference and water only works fine. Fungal growth was not a problem either.

It has been found important to ensure that the plates do not become humid or display condensation if you want to get good results. This is simply done by not over-wetting the plates, using porous micropore tape, and elevating the plates so they have air contact with all surfaces, maintaining an even temperature throughout the plate. We elevate our plates on a rack made of thin strips of wood but wire racks etc. are also suitable. Dr Botella is trialling simpler plates without vermiculite and will post the results if they are an improvement over this method.

If you want to use Basta on agar plates, see a list of protocols posted on the Arabidopsis newgroup on the 23 Sep 2000 18:57 by Anna Stepanova.

Transformation Troubleshooting

Fungal attack of the plant after infiltration: Unless you really want to transform to auxillary buds at the base of the rosette, there's no real need to immerse them. The bolt with it's flower buds are the only thing that really need to go in. Shake off as much of the infiltration media as possible to reduce the amount of sucrose coating on the plant.

The rosette leaves go yellow, wither and die in the week after the infiltration: That's normal, don't worry. It happens less if you don't immerse the rosette during the infiltration.

Not getting transgenics: The two most important things are healthy plants and sucrose in the infiltration media. Next thing to check is your Agrobacterium - are they being cured of their plasmid? Keep cultures at 28°C and avoid going over 30°C. You can also select for the virulence plasmid instead of using Rifampicin (chromosomal). This is gentamycin in

GV3101 and streptomycin in LBA4404. This is a really robust technique - not that it'll make you feel any better if you're not getting transgenics.

Hypocotyls on plants are really long: Let in some air - a complete air seal causes the plants to have long hypcotyls. I believe it has to do with too much ethylene but I'm not sure.

When do I pot the plants from the trays?: Whenever you want. I have potted out at early stages if I needed the space or left them to start to flower and then potted out. Potting out early has disadvantages. If you are choosing a representative number of lines for a construct, it can be better to wait and see what is weird. If you pot out early, you can also miss slow growing mutants. However, plants do grow more slowly in the trays and inevitably some transgenics are right next together - these can be hard to separate if you wait too long.

Get what look like transgenics but as they get older, go purple and die: You are using too much Basta. As the plants get older the nos promoter expression of the bar gene weakens and the plants die. Other ways to tell if this is the case is if trays with many transgenics look better than trays with a few transgenics on them - a large number of transgenics will deactivate the Basta in the soil better than a few so the trays with more plants can grow faster.

You aren't getting many transgenics: This one has some different possibilities. You could be using too much Basta - the bar gene is susceptible to insertion effects just like the rest of the T-DNA so insertion into a transcriptionally inactive region will mean the transgenic is not as resistant to Basta. You use just enough Basta to kill a tray of wild type plants after three sprays. You may need to do some tests here yourself.

Greatly varying efficiencies: I still get this. I think that this, just like so many other things in science, depends upon something greater like the alignment of the planets at the time.