T-DNA GENOTYPING

Vogel lab 11-7-2014

Before you start please read these notes:

1. The position of the T-DNA indicated in the database is approximate. In most cases it will be very close, but sometimes, especially for insertion sites identified using Sanger sequencing, the actual insertion site may be a few hundred bases or more away.
2. T-DNA insertions can be complex with multiple copies and pieces of the T-DNA in various orientations. This can confound PCR analysis. Therefore, it is sometimes impossible to amplify a chimeric PCR product (one primer in the gene and one in the T-DNA). In these cases you have to rely on the absence of a product from the gene specific primer (GSP) pair.
3. There is the possibility that the seeds sent do not contain the expected T-DNA insertion. This can happen if the insertion was lost in bulking due to decreased transmission or lethality. There could have also been errors in mapping the FST sequences to the correct place in the genome, labelling errors etc.
4. You will never identify a homozygous line if homozygous mutations are lethal.

SUGGESTED GENOTYPING PROCEDURE

1. Design and order gene specific primers (GSP) at least 500 bases on either side of the putative insertion site.

2. Order primers in the T-DNA (below). The T3 and R9 primers are located approximately 120 bp upstream of the 5' end of the LB (Fig 1). We have successfully used the LB primers to identify homozygous mutants for lines containing pOL001, pJJ2LBA, pJJ2LBP2, pJJ2LBP, and pJJB2LB constructs. PCR using Hyg primers (amplifies the HPTII gene on the T-DNA) can be used to detect the presence of a T-DNA insertion, but remember that there may be more than one T-DNA insertion.

Hyg Fwd	ATGAAAAAGCCTGAACTCACCGCGAC
Hyg Rev	CTATTTCTTTGCCCTCGGACGAGTGC
T3 T-DNA LB	AGCTGTTTCCTGTGTGAAATTG
R9 T-DNA LB	GATAAGCTGTCAAACATGAGAATTCAG

3. Plant out the seeds. Usually we send out about 20 seeds per line. Be sure to plant non-transgenic controls.

4. When plants are large enough extract DNA from individual plants taking care not to kill the plant. You will need seeds!

5. Perform the first PCRs with the following primer combinations (be sure to include wt and no DNA controls):

Reaction 1: GSP Fwd + GSP Rev This will produce a band when a wt copy of the gene is present. It will not produce a band for individuals homozygous for the T-DNA insertion because the T-DNA makes the fragment too large to amplify. **If you do not get a band in the wt control try new primers or optimize. Then repeat all reactions with new primers/conditions.**

Reaction 2: GSP Fwd + GSP Rev + T3 T-DNA LB This multiplex PCR should produce two bands for heterozygous individuals and single bands of different sizes for homozygous mutant or wt individuals (Fig. 1).

Reaction 3: T3 T-DNA LB This control should not produce a band. If it does you have a complex insertion. However, if the band is a different size than the bands for the GSP product or chimeric product in the other PCR reactions you can still genotype normally.

See Fig. 1 for an example where the multiplex PCR works as expected.

If you do not get a chimeric PCR product (see note 2 above) you can genotype by the presence or absence of the GSP primer band (Fig 2.). In this case, you will want to use additional control PCRs to verify that the DNA preps are ok (Fig. 2).

6. If you don't get a chimeric product and you do not identify a homozygous individual by lack of GSP band try the following reactions (all at once or some at a time):

Reaction 1: GSP Fwd + GSP Rev Same reaction as above. This will produce a band when a wt copy of the gene is present. It will not produce a band for individuals homozygous for the T-DNA insertion.

Reaction 2: GSP Fwd + GSP Rev + R9 T-DNA LB This multiplex PCR uses the other T-DNA primer and should produce two bands for heterozygous individuals and single bands of different sizes for homozygous mutant or wt individuals. Sometimes one LB primer works better than the other.

Reaction 3: R9 T-DNA LB This control should not produce a band. If it does you have a complex insertion. However, if the band is a different size than the bands for the GSP product or chimeric product in the other PCR reactions you can still genotype normally.

The following reactions are non-multiplexed PCRs to amplify a chimeric product. Try all combinations in case the T-DNA orientation is not as predicted. Sometimes a non-multiplexed PCR works better than the multiplexed reaction.

Reaction 4: GSP Fwd + R9 T-DNA LB

Reaction 5: GSP Rev + R9 T-DNA LB

Reaction 6: GSP Fwd + T3 T-DNA LB

Reaction 5: GSP Rev + T3 T-DNA LB

If you get chimeric PCR product you can genotype. If you find only heretozygous individuals (as you would if the first set of PCRs were inconclusive) you will have to genotype the progeny of the heterozygous individuals to try and find a homozygous mutant. Remember, if the mutation is lethal you will never get a homozygous mutant.

If none of the chimeric PCRs work and the GSP product is never missing you have one of the following problems:

A. Problem: The insertion is complex so you will never get a chimeric PCR product and you did not find a homozygous individual (missing GSP band) because you are unlucky or the mutation causes decreased transmission.
   Solution: Genotype many progeny from the lines you tried to genotype.
B. Problem: The homozygous mutant is lethal and the chimeric PCR does not work.
   Solution: Examine the segregation ratio of the transgene using the Hyg primers in the initial lines and their progeny. If there is an insertion at one locus and it is lethal when homozygous you expect the Hyg resistance to segregate 2:1 instead of 3:1 and you will never get a line homozygous for Hyg. Be aware that there may be more than one T-DNA insertion in the lines and this can complicate the analysis.
C. Problem: The insertion is further than 500bp from the predicted insertion site.
   Solution: Try genotyping with primers 1,000 bp away from the insertion site if desired.
D. Problem: The line does not contain the expected insertion for any of a number of reasons (note 3).
   Solution: Consider another method to get a mutation like CRISPR/Cas9 or TILLING.

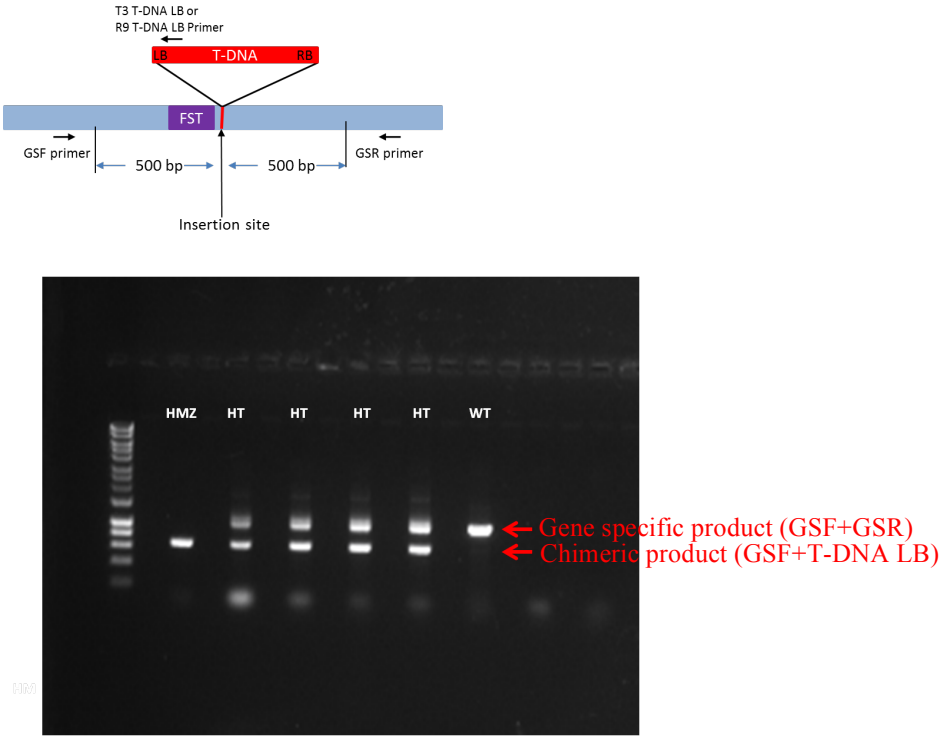

Figure 1. Genotyping example multiplex PCR
Diagram of T-DNA insertion showing the location of the primers. FST is the sequence recovered when the T-DNA was sequenced. The gel image shows six T_2 individual plants from a T-DNA line genotyped using GSP fwd + GSP rev + T3 T-DNA LB primers. The T_2 population is still segregating for this T-DNA insertion. The GSPs are designed to produce an approximately 1000 bp product. In these reactions, homozygous individuals are identified by the absence of the 1000 bp product (the T-DNA insertion makes the fragment too large for PCR) and the presence of a chimeric product of about 600bp.

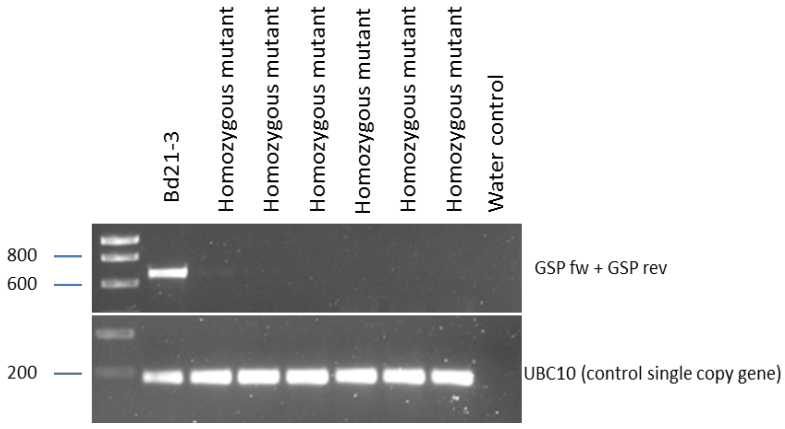

Figure 2. Genotyping with the gene specific primers. The top gel shows a wt individual (Bd21-3) and 6 homozygous mutants from a previously genotyped homozygous plant. The water control checks for DNA contamination. Note that homozygous mutants do not have a band from the GSP pair. The lower gel shows the amplification of a single copy gene as a control for DNA quality.