Genotyping protocol - Chloroplast 2010 Project

Overview:

- Genotyping is done in sets corresponding to each grow-out: either a grow-out that was done for leaves (a screen, i.e. Screen #1) or to get seeds (bulk seed, i.e. BS#20). Each set consists of 192 individual samples (maximum) corresponding to 2 siblings of 96 alleles (i.e. SALK_123456). Most of the time there are far fewer than 192 to genotype due to non-SALK mutants present that cannot be genotyped, wt plants, or plants that did not survive.
- Primers are designed using the SALK T-DNA Primer Design Tool, http://signal.salk.edu/tdnaprimers.html, with the Ext5 value changed to 200 because LBa1 is the T-DNA primer we use for 2nd pass reactions; otherwise the products would be too long.
- Primers are manufactured in plates at IDT, 10 nmoles per well, dry, with all LP in one plate, and all RP in the other, in alphanumeric order by ascending SALK number for that set. PCR plate designs files are set up so PCR plates can be filled with primers from diluted plates quickly and accurately using multichannel pipettors.
- "First pass" reactions are done using a condensed experimental design that distinguishes homozygous alleles for both siblings from any non-homozygotes, then more extensive "2nd pass" reactions are done to distinguish between heterozygotes and wild type alleles for the samples that test positive for presence of a wild type allele.

"First pass" genotyping:

- Each of two sibling samples are placed in the same reaction well to genotype with 2 specific, genomic primers (LP & RP). Placing the two siblings together saves LOTS of plates.
- One wt sample is also genotyped in the adjacent well with the same 2 specific, genomic primers, as a positive control
- Samples are amplified using ABI SYBR Green PCR Master mix (cat#4309155), and read at the RTSF as an RT-PCR end-point read at 72°C (so that agarose gels do not have to be run for all the samples, saving LOTS of supplies and time). ABI SYBR Green master mix is "fortified" by adding 1 unit Ampli-Taq Gold per 15 ul Master Mix (1 unit of extra Taq enzyme per reaction) to enhance the PCR reaction. Primers are added to a final concentration of 0.1 uM.
- Cycling conditions for SYBR Green master mix:
  10 min 95° (initial enzyme activation)
  2 min 95°
  1 min 56°
  2 min 72°
  (repeat for 38 cycles)
  10 min 72°
  Hold at 4°
- Interpreting results:
  - If the two sibling punches do not yield a PCR product (a band on a gel or a SYBR-Green average value >0.15 via RT-PCR end-point read) but the wt sample does, then both siblings are called as homozygous (neither contains any wild-type DNA for that

gene). ***Note that this is a rapid assay and occasionally will yield false negative results.***

- If the well containing the two sibling punches yields a wt-sized (same as wt sample) band, then at least one of the samples is either heterozygous or wt for the intended T-DNA insertion. These samples must then be genotyped in the "2nd pass."
- If the wt sample does not yield a PCR product, then something is wrong with the primers or reaction; repeat in "2nd pass." Occasionally, new primers will need to be designed to get a result.

<u>"Second pass" genotyping</u>:

- Sibling are analyzed in separate wells. Each sibling (DNA Card sample) is amplified using two separate reactions: one with the gene-specific genomic primers LP & RP, and one using RP + LBa1, an insert-specific primer which will yield an amplified fragment only if the insert is present.
- One wt sample is also genotyped in the adjacent well with the same LP & RP, as a positive control.
- Samples are amplified using Redtaq Readymix (Sigma P2523) and primers to a final concentration of 0.1 uM, and analyzed by visualization on agarose gels.
- Cycling conditions for Redtaq Readymix:
  2 min 95° (initial melting)
  2 min 95°
  1 min 56°
  2 min 72°
  (repeat for 34 cycles)
  10 min 72°
  Hold at 4°