Designing crossing schemes

Once you have selected mutant lines for your gene(s) of interest, there are a number of ways in which you can proceed depending on the aim of your study. Here we describe a couple of common use scenarios, though this is by no means an exhaustive list. We provide training documents on "How to grow wheat", "DNA extraction" and "Crossing wheat" if you aren't familiar with these procedures.

a) Investigating effects of a single gene

In some cases the aim of the study maybe to look at the effects of knocking out a single gene. As each mutant line contains several thousand mutations, backcrossing to Wild Type (WT), either Kronos or Cadenza depending on the population used, will be required to reduce the number of background mutations.

The first step is to screen the mutant seeds you have received to confirmed that they contain the mutation. This can be done using KASP genotyping or conventional Sanger sequencing using the pre-designed Polymarker primers if available or designing genome specific primers. The minimum number of seeds you should screen will depend whether the mutation is homozygous (hom) or heterozygous (het). Fewer seeds need to be screened if you have a hom mutation as, in theory, the mutation should be fixed and present in all seeds. Heterozygous mutations will most likely still be segregating in the M_4 or M_5 seed so a larger number of seeds will need to be screened to identify homozygous mutations. It is important to consider that some of the mutations that were heterozygous in the M_2 (the sequenced generation) may have been fixed in the M_4 or M_5 seed, whereas other mutations may have been lost through genetic drift. A suggested number to start with could be 4 seeds for a hom mutation and 8 for a het mutation, but more seeds should be screened if necessary. It is preferable to select individuals that are homozygous for the mutation to take forward for crossing but heterozygous individuals can be used if necessary.

The next step is to carry out a number of rounds of backcrossing to WT followed by self-pollination to obtain plants that are homozygous for the mutation of interest whilst reducing the number of background mutations. Each round of backcrossing and selecting specifically for the mutation of interest will reduce the number of background mutations by approximately XX%. In this way you could effectively produce Near Isogenic Lines (NILs) with and without the mutation of interest and compare the phenotype of these.

Alternatively, after the first backcross (BC_1) a heterozygous individual could be self-pollinated to create an F_2 population segregating for the mutation in which an initial phenotype could be assessed. Phenotyping and genotyping of this F_2 population will give an initial insight as to how the mutation is associated with your phenotype of interest. These two approaches can be carried out in parallel.

It is important to remember to include the appropriate controls. This could be the WT line (either Cadenza or Kronos, depending on the population from which the mutant line is from. Alternatively, WT individuals could be selected alongside mutant individuals during the backcrossing process to generate more specific experimental controls. A heterozygous mutant can actually provide a proper

experimental control in case you want to assess the phenotype in this first generation as there is the opportunity to identify both homozygous mutant and wild type plants in the seeds you receive.

NB. whilst phenotypes maybe visible for single knock outs of certain genes this will not be the case for all genes as there is a high level of functional redundancy in the polyploid genome of wheat.

b) Creating complete knockouts

In many cases, it may be necessary to look at complete knock outs of all homoeologues. In these situations, it is more straightforward to use the Kronos (tetraploid) population as only a double mutant needs to be generated (A + B genome). However, if you have to use the Cadenza population (e.g. you can only find Cadenza stop mutations, or you wish to study the effect in an elite hexaploid background) triple mutants can be generated (A + B + D genome).

As above, the first step is to screen seeds to identify homozygous individuals (but in this case for each mutant line identified for the A and B genome). Homozygous individuals for A genome and B genome mutations should then be crossed together and the progeny screened for a variety of mutations. Ideally you are looking for individuals that have both the A genome and B genome mutations (these will be het in this generation). However, it may also be of interest to take forward individual mutants and WT individuals for comparison. The next step is to self-pollinate the selected individuals to generate homozygous double (and single, if required) mutants. This F_2 population could also be phenotyped to get a first insight into the effect of the mutations. Once homozygous mutants have been identified backcrossing to WT can be carried out to reduce the number of background mutations and NILs generated if desired. If you want to create a triple mutant, the D genome mutation should be introduced at this stage by crossing the homozygous double mutant with a homozygous D genome mutant. Selection and self-pollinate should then be carried out in the same way as described above but looking for triple mutants and other combinations of mutations as required.

Above, we describe two of the most common crossing schemes that may be used with TILLING mutant lines. Other potential use scenarios could include crossing together mutations in different genes in a particular pathway or crossing the mutations into other backgrounds e.g. crossing a Cadenza mutation into another elite hexaploid line. In all cases the same basic principles apply: it is critical to confirm the mutation and its zygosity in the seeds you receive, you must take into account the effect of background mutations and ideally try to reduce the load, and you must include the appropriate controls when assessing phenotypic effects.

It is also important to take into consideration how confident you are of the effect of the mutation in your gene of interest: a stop mutation early in the gene is a good indication that the gene will be non-functional, whereas the effect of missense mutations are harder to predict. See "Selecting TILLING mutants" for more information on these considerations.