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", "Speed Breeding", "Crossing wheat" and "DNA extractions" if you are not familiar with these procedures. More information on strategies to use the mutant lines is available in Uauy *et al.* 2018 Annual Review of Genetics.

a) Investigating effects of a single gene

In some cases, the aim of a study may be to investigate the effects of knocking out a single gene. As each mutant line contains tens of thousands of mutations, backcrossing to the recurrent wild-type (WT), either Kronos or Cadenza depending on the population used, is useful to reduce the number of background mutations.

The first step is to screen the mutant seeds you have received to confirm that they contain the expected mutation(s). This can be achieved via KASP genotyping or conventional Sanger sequencing using pre-designed primers from PolyMarker; alternatively, you can design your own genome-specific primers. The minimum number of seeds you should screen will depend on whether your mutation is homozygous (hom) or heterozygous (het). Fewer seeds need to be screened if you have a homozygous mutation as it should be fixed and present in all seeds. Heterozygous mutations will most likely still be segregating in the M_4 or M_5 seed and thus a larger number of seeds should be screened to identify homozygous mutants.

It is important to remember that the mutations were originally called on M_2 individuals; it is therefore possible that mutations which were heterozygous in the M_2 are now either homozygous or completely lost in the M_4 or M_5 seed. A suggested number to start with could be 8 seeds for a homozygous mutation and 16 seeds for a heterozygous mutation, but more seeds can 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; when crossing with heterozygotes you need to make twice as many crosses as with homozygotes to obtain sufficient F_1 seed.

The next step is to carry out multiple rounds of backcrossing to the WT, followed by self-pollination, to obtain plants that are homozygous for the mutation of interest with a reduced load of background mutations. Each round of backcrossing and selecting specifically for the mutation of interest will theoretically halve the number of background mutations. In this way you could effectively produce Near Isogenic Lines (NILs) with and without the mutation of interest and compare their phenotype.

Alternatively, after the first backcross (BC_1), a heterozygous individual could be self-pollinated to create an F_2 population segregating for the mutation; this would allow you to perform a preliminary assessment of the segregating phenotype. Geno- and phenotyping this F_2 population will give an initial insight into the effect of the mutation on your phenotype of interest. Both approaches can be carried out in parallel.

It is important to remember to include the appropriate controls! The best control when phenotyping TILLING lines are WT individuals descended from the original mutant line. These may be derived directly from the M_4 or M_5 seeds, when dealing with a heterozygous mutation, or from a BC_1F_2 segregating population, for example. If this is not possible, the WT line parent (Cadenza or Kronos) can be used, though these will not be comparable to the mutant lines until after multiple generations of backcrossing.

NB: Whilst phenotypes may be visible for single knockouts of certain genes this will likely not be the case for most genes due to the high level of functional redundancy in the polyploid genome of wheat.

b) Creating complete knockouts

In many cases it will be necessary to create complete knockout lines to observe a mutant phenotype. If your aim is to screen a list of candidate genes, it is easier to use the Kronos (tetraploid) population to validate them; using the Kronos population requires only a double mutant to be generated (A + B genome) for a full knockout line. If you must use the Cadenza (hexaploid) population, because you cannot find premature STOP mutations in the Kronos population or because you wish to study your gene in a hexaploid background, you need to create triple mutants (A + B + D genome).

Let us first consider creating a complete knockout using tetraploid Kronos lines. The first step is again to identify homozygous mutants for each of the homoeologous copies of your gene of interest. Individuals with homozygous mutations in the A- and B-genome copies of your gene of interest should then be crossed and the progeny screened for all mutations; based on Mendel's first law all F_1 individuals should be heterozygous for the A- and B-genome mutations. The next step is to self-pollinate the selected individuals to generate homozygous double (and single, if required) mutants. At this stage, the F_2 population can be phenotyped for a preliminary assessment of the effect of the mutations. Simultaneously, the homozygous double mutants can be backcrossed to the WT parent to reduce the number of background mutations and to create NILs, if desired.

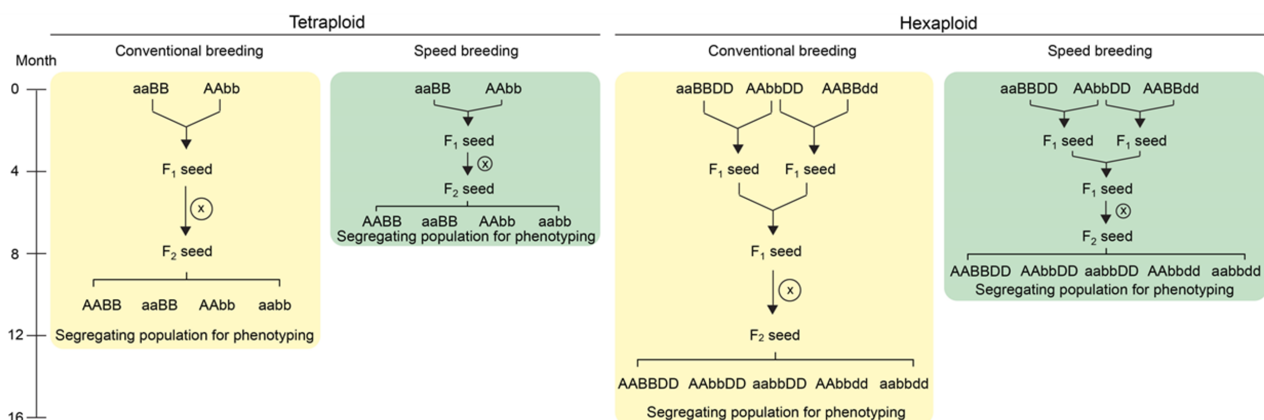

Figure 1: Crossing scheme to combine TILLING single mutants. In tetraploids, mutations in the A and B genome homoeologs of a gene of interest can be combined through a single cross. The F_1 plants are self-pollinated to produce a segregating F_2 population which contains homozygous double and single mutants, as well as wild type plants (screening by PCR required; only four genotypes shown). These F_2 progeny can be characterised for preliminary assessment of the phenotype of interest. The use of speed breeding reduces the time taken

to reach this phenotyping stage from 12 (yellow) to 7.5 months (green). In the hexaploid population a second round of crossing is required to combine the three mutant alleles. The F_2 progeny segregating for the three mutant alleles can be genotyped using PCR to select the required combination of mutant alleles (only 5 genotypes shown; all factorial combinations are possible). Speed breeding reduces the time taken to generate triple homozygous mutants for phenotyping to 10 months (green), compared to 16 months in conventional conditions (yellow). Self-pollination is represented by an X inside a circle. Figure from Adamski et al. 2018 *PeerJ*.

To create full knockout mutants in the hexaploid Cadenza background, you can follow a similar scheme. Having identified homozygous mutants of all three homoeologs, you can make two crosses: the first one, as above, to combine A- and B-genome mutations and the second one to combine B- and D-genome mutations. Verify the presence of all mutations in your F_1 plants and let them self-pollinate. Identify homozygous double mutants for both sets of crosses in the F_2 and make a new cross from them. As the B-genome mutation was homozygous in both crosses, it will be already fixed in your new F_1. This means that you can expect 1 in 16 F_2 plants to be a homozygous triple knockout. If you would cross the three mutations one after the other, this ratio would go up to 1 in 64 lines.

Here, we have described two of the most common crossing schemes that may be used with the TILLING mutants. But there are other ways that these populations can be used for: you could combine mutations in genes belonging to specific pathways, e.g. starch biosynthesis or backcross the mutants into another (adapted?) background line. In all these cases the same basic principles still apply:

1) it is critical to confirm the mutation and its zygosity in the seeds you receive
2) take into account possible effects of background mutations on your phenotype and ideally try to reduce the background mutation load
3) you must include 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 premature 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 mutations" for more information on these considerations.