Medicago truncatula hybridization – supplemental videos

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1. Introduction

Since its adoption by the scientific community as a model legume, *Medicago truncatula* has proven an invaluable resource for information in a variety of areas from basic studies on pathogenic and mutualistic interactions, to mining the genome for "genes of interest", to metagenomic analysis and bioinformatics computational modeling. Various established mutant populations (e.g. obtained via fast neutron bombardment deletion or retrotransposon insertion) now allow researchers to isolate particular loss-of-function genes in order to observe associated phenotypic variations. However, lines with disruptions in multiple genes can lead to difficulties in ascribing attributes to a single or specific gene. The removal of unwanted disruptions necessitates several backcrosses to the parental line. Cross-hybridization also provides a way of combining mutations from two (or more) separate lines as well as transferring a transgene from a donor line.

The three instructional videos presented here were created independently over several years (2006-2009) in order to document methods of cross-hybridizing *Medicago truncatula*. They are presented here to supplement the method described in the *Medicago truncatula* Handbook chapter Vernalization, crossings and testing for pollen viability (Chabaud *et al*., 2006). The first two videos are appropriate when working in either the A17 or R108 background. The third video is so far only relevant to the A17 background as it deals with using the male sterile *tap* line (for *M. truncatula apetala*) (Penmetsa & Cook, 2000). A clear understanding of flower architecture is required for the process of reproductive manipulation described here. Figure 1 has thus been added to be used as a platform for discussing *in situ* flower dissection for the purpose of accessing and manipulating the reproductive system. Figure 1 illustrates the *Medicago truncatula* flower architecture and petal arrangement at maturity, during that window of time when cross-hybridization can be affected.

One helpful feature of *M. truncatula* is that it self-fertilizes, a process occurring inside the flower before it opens. This allows for controlled breeding and offers a ready opportunity for cross-hybridization, such as backcrossing, followed by selfing for segregation analysis. This same feature also complicates gaining access to the reproductive column and manipulation of the fertilization process. By opening the "female" flower (Pollen Recipient) prior to pollen release, the immature anther sacs can be removed and the stigma fertilized with pollen obtained from another "male" flower (Pollen Donor), also opened before the opportunity for pollen contamination from

other sources. When working in the A17 background, there is an additional crossing tool available, the male sterile *tap* mutant, which forms an abnormal flower lacking functional stamens (anthers). Here hybridization simply involves the application of pollen from a male donor to the exposed [female] stigma.

Figure 1 - Mature *Medicago truncatula* flowers showing petals and reproductive column.
A - Standard petal as mounted for dissection. **B** - Standard petal folded back exposing wing petals and keel petal. Note the reproductive column at basal opening between wing petals and keel petal.
C - Standard petal folded back, wing petals and keel petal spread, showing the [fused] reproductive column. Note the released pollen surrounding the stigma (Pollen Donor). **D** - Reproductive column with stigma surrounded by [immature] anthers (Pollen Recipient).

2. Materials
Female "recipient" plants
Male "donor" plants
Dissecting microscope (with 20x capability)
Tape
Two pair extra-fine forceps (Dumoxel Biology – Dumont #7)
Fine scissors
Scalpel
Magnifying visor (Bausch & Lomb, 2.6 magnification)
Self-adhesive labels
Perforated plastic sheets (Chantler Packaging)

3. Methods

The criteria for selection of female and male flowers are covered extensively in the Handbook. Described here are the salient features of the procedures shown in the videos as well as mention of issues encountered in trying various methods and reviewing the literature. Once the plants have started forming pods, crossing is best done under a dissecting microscope. Standard lighting for stereomicroscopes consists of "cold light" from a halogen lamp plus optic fibers. If this is not available ambient lighting is acceptable. Normal “hot” under-stage lamps in dissecting microscopes will desiccate flowers and are therefore not advised.

Center and tape the [female] flower to the microscope stage on its side (Fig. 1A). The standard petal can be opened either by slicing down through both layers just below (~1 mm) the midline and removing the resultant strip (Video 1) or by slicing along the midline itself (Video 2). Next the standard petal, wing petals and keel petal are spread apart to allow access to the fused reproductive column (Figs. 1C and 1D, Figs. 2B and 2C). Emasculation, or removal of the immature anther sacs (Fig. 1D and Fig. 2C), may be accomplished using forceps or vacuum aspiration. If pollen has already been released (Fig. 1C), discard the flower and start over.

Male flowers can be harvested ahead of time and stored in water or the sexual columns kept on moist filter paper until needed (up to 1 hr) (Chabaud *et al*., 2006) or selected and used directly following emasculation. Mature reproductive columns curl upward, or "trip", when the restraining flower petals are removed. In other members of the Leguminosae family the tripping mechanism effectively strikes the stigma against insects looking for nectar. This action disperses pollen and ruptures the hyaline membrane surrounding the stigma, a prerequisite for fertilization (Armstrong & White, 1935). The ruptured membrane not only allows penetration by germinating pollen, but releases a critical viscous fluid which "probably aids pollen germination" (Lesins & Lesins, 1979). Although the *M. truncatula* flower self-pollinates prior to flower opening and any obvious tripping, the dynamics of membrane rupture may well have a bearing on cross-hybridizing success. Tripping makes harvesting pollen a bit tricky. Using a magnifying visor and two forceps, hold the flower at its base with the crescent pointing up and away from you. Carefully peel the standard petal back and remove it. Insert the forceps tip between the keel and wing petals until gently touching the reproductive column (Fig. 1B). Slide the forceps towards the tip of the column spreading the petals while preventing the column from tripping at the same time. Slowly allow the column to curve upwards. With practice the apex of the column will sport a tuft of mature pollen (Fig. 1C).

Back under the dissecting scope, with the forceps, carefully turn the female flower so the stigma is pointing up (Fig. 1D, Figs. 2B and 2C). Hybridization is accomplished by bringing the male donor pollen down over the female flower's stigma and slightly wiggling the column to apply the pollen (applying and positioning the pollen may be aided with forceps). When properly done you get a tuft of pollen surrounding the stigma. Gently remove the flower from the stage, place an adhesive label on the internode just below the flower and record date and male donor identifier.

Post-hybridization, the flower may be left as is (Video 1), placed in an inverted tube and sealed with moistened cotton (Videos 2 & 3) or in a vial containing water and sealed with cotton for 48 hr (Chabaud *et al*., 2006). Wait until the developing pod is 2-5 days old and has 2-3 complete coils. At that point wrap the pod [in perforated plastic sheeting fastened with staples] to prevent pod loss. Remove any new leaves and branches to prevent vegetative growth within the plastic wrap, allowing normal apical growth.

The third video presents an alternative method, using male sterile plants with flowers having no petals and no functional anthers. In this case the male sterile flower is the pollen recipient and requires a fully functional flower as a pollen donor.

4. **Conclusions**

These three videos on crossing *Medicago truncatula* are presented as supplemental to the methods presented in the *Medicago truncatula* Handbook in the chapter Vernalization, crossings and testing for pollen viability (Chabaud *et al*., 2006). Although the original method and video (to be completed) depict the optimum procedure, a number of variants of steps along the way can produce acceptable results, depending on individual circumstances, needs, and temperament. Variations in technique support a central theme of opening the [female] flower, emasculation, pollen harvest, and cross-pollination. Also presented here is the use of a male sterile flower, which simply requires pollen harvest and cross-pollination. In all cases the relative maturity level of the flowers involved is critical.

The main difference between the procedures presented here and those of Chabaud *et al*. is in the preparation of the female flower. Here we open flowers down the midline of the standard petal to gain access to the reproductive column. In the Chabaud method, the flower is opened 1/4 - 1/3 of the way down to one side from the midline of the standard petal, creating a protective pouch into which the stigma is placed after pollination (Fig. 2; E.-P. Journet, personal comm.).

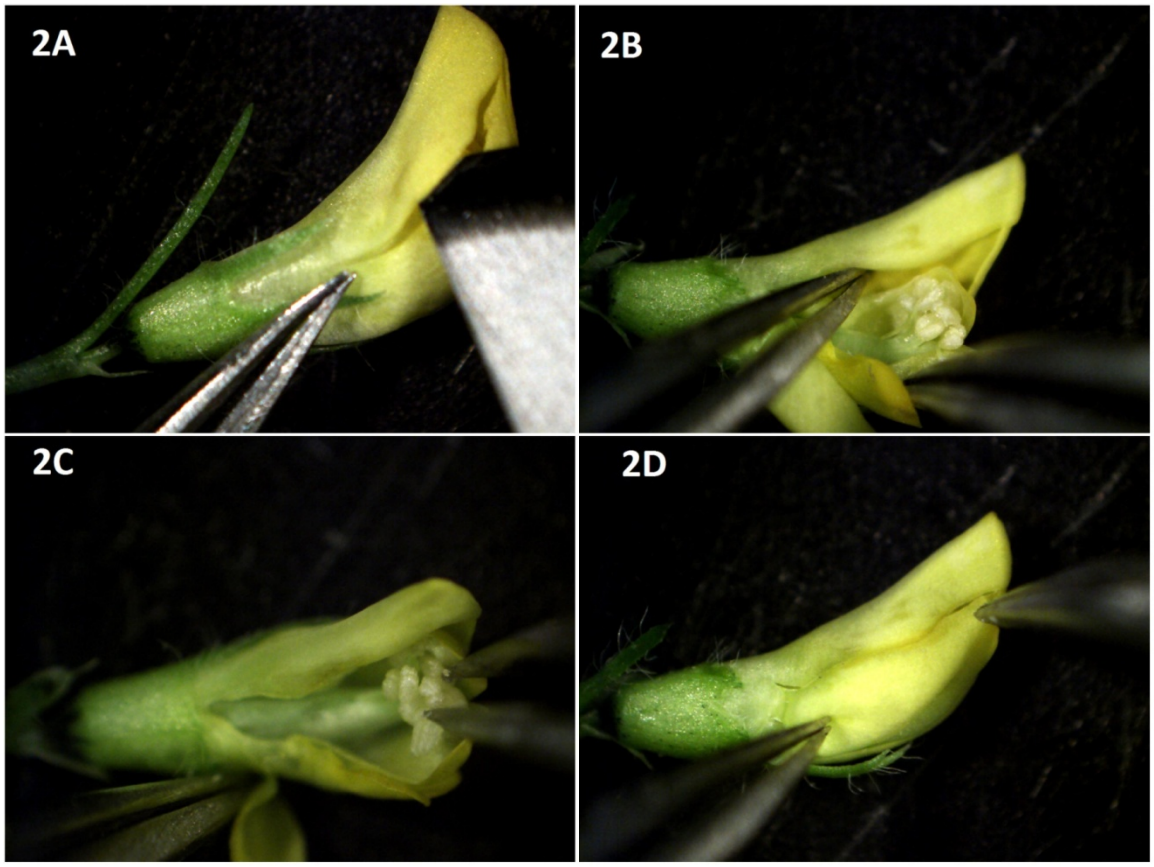

Figure 2 - Pollen recipient flower undergoing the pouch method of cross-hybridization (Chabaud *et al*., 2006). **A** – Flower mounted and receiving initial incision. **B** – Standard petal pouch, wing petals and keel petal spread, allowing access to the reproductive column. **C** – Removal of [immature] anther sacs, to be followed by pollination. **D** – Flower closed back around cross-hybridized reproductive column.

For the novice, the dexterity involved in accomplishing the pouch method can be a bit daunting and time consuming. The midline opening is significantly more achievable until a higher level of proficiency is attained. It should be emphasized here that the original pouch method significantly increases crossing success rates, and also number of seeds per pod, and has become standard operating procedure for experienced individuals.
The methods presented collectively in the four videos offer a range of options and alternatives for the steps involved in the process of cross-hybridizing *Medicago truncatula* for both the beginner and the advanced practitioner. As we go through the key aspects of these procedures, there are several options for any given task. Although removal of surrounding flower buds may facilitate development of the crossed flower, removal of too much surrounding vegetation (leaves and shoots) may traumatize the plant and should be avoided. Minimal disturbance may be the best approach. Certainly when opening a "female" flower, care should be taken to minimize damage to petals, reproductive column and the entire pistil during manipulations. Pollen harvest, by squeezing the flower at its base, peeling away petals with a magnifying visor or under a dissecting microscope, allows for personal preference, as does applying pollen, with a male column or with forceps. Post-hybridization, there are various ways to host the flower until pod development has begun. Customize your procedures as it suits your circumstances and ability. Beyond the act of placing pollen on the stigma, the best strategy for success is to re-pack the pollinated reproductive column back into the flower petals as close as possible to the original position and close the standard petal pouch over it all, ensuring a natural environment for pollen germination and fertilization to occur.

5. Link to Videos
https://www.noble.org/medicago-handbook/videos/

6. Acknowledgements

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7. References

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