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Low-cost methods for molecular characterization of mutant plants: from tissue collection to mutation discovery

For the July 1-5 Training Course on "Plant Mutation Breeding: Mutation Induction, Mutation Detection and Pre-Breeding"

Plant Breeding and Genetics Laboratory, June 2013

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How to use this training course protocol: The PBGL has developed detailed and low-cost protocols for many different molecular assays. Due to the short time that we can schedule molecular techniques, not all steps can be taught in this course. Students will perform the activities in blue. Background information and additional protocols are provided. The PBGL has developed many protocols not listed here. Please contact us for a full list of methods developed.

1: Introduction

1.1 Background

A variety of molecular methods can be employed for the characterization of natural and induced nucleotide variation in crop plants. These facilitate a better understanding of gene function and allow a reduction in the time to breed new mutant varieties. Molecular biology, however, can be difficult to master and while efficient, many protocols rely on expensive pre-made kits. The Plant Breeding and Genetics Laboratory have been developing a series of low-cost approaches for the molecular characterization of mutant plant materials. In this course, students will learn methods common to many molecular analyses, namely proper collection and storage of plant tissues and extraction of DNA. Also, students will learn low-cost TILLING and Ecotilling approaches for the characterization of single nucleotide mutations and indel variation in plant genomes.

The extraction of high quality and quantity genomic DNA from tissues is at the heart of many molecular assays. Indeed, with the routine use of molecular markers and more recently the application of next generation sequencing approaches to characterize plant variation, recovery of DNA can be considered a fundamental tool of the plant scientist. The basic steps of DNA extraction are: 1) Proper collection and storage of plant tissues, 2) Lysis of plant cells, 3) solubilisation of lipids and proteins with detergents, 4) separation of DNA from other molecules, 5) purification of separated DNA, and 6) suspension in an appropriate buffer. Isolation of DNA dates to the late 1800s with the work of Friedrich Miescher and colleagues who first discovered the presence of DNA in cells long before it was established that DNA was the genetic material (DAHM 2005).

1.2 *Methods used to isolate genomic DNA from plant tissues*

The advent of recombinant DNA technologies and DNA sequencing technologies in the 1970s marked the beginning of a rapid expansion of molecular biology analyses in plants that continues. In parallel, DNA isolation procedures tailored to the unique aspects of plant cells have evolved. A variety of DNA extraction methods have been described, however there are several methods most commonly used by plant biologists. One of the most enduring methods in plants is the so called CTAB method because a main component of the lysis buffer is cetyltrimethylammonium bromide (CTAB), which solubilizes membranes and

complexes with the DNA. First described in 1980, the method employs an organic phase separation using a chloroform-isoamyl extraction, and alcohol precipitation to isolate DNA from proteins and other materials (MURRAY and THOMPSON 1980). The method remains popular in part due to the fact that all components can be self-prepared and thus the cost per sample remains low. Wide and prolonged usage of the method also validates the approach for many different molecular assays. However, manual phase separation means that human error can introduce unwanted cross-contamination of organic compounds that may result in an inhibition of downstream enzymatic assays. Further, chloroform is a toxic organic compound and proper ventilation and waste disposal measures are needed.

An alternative to the CTAB method is the use of high concentrations of potassium acetate and the detergent sodium dodecyl sulfate (SDS) (DELLAPORTA *et al.* 1983). Proteins and polysaccharides are precipitated and removed from the soluble DNA. This approach is advantageous to the CTAB method in that organic phase separation is avoided. However, a filtration step may be required to remove plant material and precipitated from soluble DNA, limiting the throughput of the method.

In recent decades, commercial kits for the rapid extraction of DNA from plant material have become routine. There are many examples; however popular kits include those sold from Qiagen and MP Biomedicals (www.qiagen.com/, www.mpbio.com/). Commercial kits have proven to be highly reliable in producing high yields of highly purified DNA, and so have become the standard when performing sensitive molecular assays. Many such kits utilize the binding of DNA to silica in the presence of chaotropic salts. In the presence of a high concentration of chaotropic salt, the interaction of water with the DNA backbone is disrupted and charged phosphate on the DNA can form a cationic bridge with silica while other components remain in solution. Silica is either used in a solid phase as in spin columns, or in slurry for batch chromatography. Washing the DNA bound silica in the presence of a high percentage of alcohol removes excess salt. Addition of an aqueous solvent (water or buffer), drives the hydration of the DNA and the subsequent release from the silica. The now soluble DNA can be separated from silica through a quick centrifugation step. The method is rapid, taking less than one hour, and is scalable such that high-throughput 96 well plate assays are common. While highly advantageous over other methods, such kits remain expensive when compared to home-made methods such as CTAB and Dellaporta approaches. Therefore, the

methods described here were developed to provide the ease and quality of silica based DNA extraction at a fraction of the cost.

1.3 *Methods for the discovery and characterization of induced and natural nucleotide variation in plant genomes.*

Nucleotide variation is the major source of phenotypic diversity that is exploited by plant breeders. Variation can be either natural or induced. In the late 1990s a reverse-genetic strategy was developed whereby induced mutations were used in combination with novel methods for the discovery of nucleotide variation (MCCALLUM *et al.* 2000). Known as TILLING, for Targeting Induced Local Lesions IN Genomes, the approached allows for the recovery of multiple new alleles in any gene in the genome provided the correct balance of population size and mutation density can be achieved (COLBERT *et al.* 2001; TILL *et al.* 2003). Efficient methods for the discovery of Single Nucleotide Polymorphsism (SNP) and small insertion/deletions (indels) were developed utilizing single-strand specific nuclease that can be easily prepared through extractions of plant materials such as celery, or mung beans (TILL *et al.* 2004a). TILLING has been applied to over 20 crop species, and similar methods have been used to characterize natural nucleotide variation, known as Ecotilling (COMAI *et al.* 2004; JANKOWICZ-CIESLAK *et al.* 2011). While TILLING and Ecotilling have been primarily used in seed crops, the methods work well in vegetatively propagated and polyploidy species such as banana (JANKOWICZ-CIESLAK *et al.* 2012; TILL *et al.* 2010).The PBGL has developed low-cost methods for the extraction of enzymes from a variety of plant materials, including collections of weedy plants. The PBGL has also developed low-cost agarose gel based methods for TILLING and Ecotilling assays. In this training course, students will use low-cost mutation discovery assays to identify nucleotide variation in crops.

2: Health and Safety considerations

All laboratories should have standardized health and safety rules and practices. These can vary from region to region due to differences in legislation. Before beginning new experiments, please consult your local safety guidelines. Failure to follow these rules could result in fines or a closure of the laboratory. Consider the following guidelines applicable to all laboratories:

1. Always wear a laboratory coat in the laboratory. Remove the coat when exiting the lab to avoid contaminating people with the things you are protecting yourself from.
2. Wear eye protection (specialty safety goggles) when working with chemicals or anything that you don't want entering your eye!
3. Wear gloves to protect your hands from dangerous materials, and to protect your samples from contamination. Do not touch common items like the telephone, door handles, or light switches, with gloves as the next person touching those items may not be wearing gloves. Remove gloves before leaving the laboratory.
4. Wear proper foot protection, and avoid open toe footwear.
5. Wear clothing that covers your legs. Avoid loose fitting clothing that can be caught in machinery or pass over open flame.
6. Familiarize yourself with emergency procedures. Know where the nearest eyewash station and shower is located. Know where the nearest first aid kit is located, and locate the list of emergency telephone numbers.
7. Consult the Materials Safety Data Sheet (MSDS) for the chemicals you will be using. These sheets should come with the chemicals. They provide health risks, first aid measures, fire and explosion data, how to deal with accidental release (spills), handling and storage, and guidelines for personal protection. If you don't have the MSDS, you can find them by doing a web search of the item with MSDS in the title. For example, searching for "MSDS Hydrochloric

acid" using Google, results in a series of links to the MSDS sheets provided by commercial suppliers of hydrochloric acid.

8. Locate the emergency spill kit to handle accidental spillage of hazardous materials. If your laboratory is not equipped, consider preparing a kit (see Appendix 1).
9. Don't rush. If you are unfamiliar with a piece of equipment, or concerned about the safety of a procedure, stop! Make sure you know what you are doing and the risks associated with the procedures before you begin.

3: Sample collection and storage

Of key importance to the successful extraction of genomic DNA from plant tissues is the collection of suitable material and proper storage of the tissues or cells before DNA isolation. If the samples are not properly treated, DNA can be degraded before isolation procedures are commenced. The rate of sample degradation can vary dramatically from species to species depending on the method of sample collection. Mechanisms of degradation of genomic DNA include the activity of nucleases that can be exposed to nucleic acid upon organellar and cellular lysis. Therefore, a common method is to isolate leaf or root tissues and flash freeze in liquid nitrogen, followed by prolonged storage at -80°C. At these temperatures, nucleases remain inactive and DNA is stable. Thawing of tissue in some species can lead to rapid degradation. Therefore, during the extraction procedure, it may be necessary to immediately expose frozen tissue to a lysis buffer containing EDTA, which inhibits nuclease activity.

While collection of tissues in liquid nitrogen and -80°C storage may be highly suitable for most plant species, it can be impractical in some developing countries because liquid nitrogen can be expensive and hard to procure, and due to the expense of providing continuous power supplies for -80 freezers. Lyophilisation, or freeze drying, is an alternative approach that results in tissue samples that can be stored at room temperature for many months prior to isolation of DNA. This has been used to produce high quality genomic DNA suitable for high throughput TILLING assays (TILL *et al.* 2004b). Lyophilisation circumvents the need for continual -80C storage, but commercial lyophilizers can be expensive. An alternative method is storage of collected tissue in silica (CHASE and HILLS 1991; LISTON *et al.* 1990). This removes water from tissues and in many cases the dried material is stable at room temperature for weeks to months before isolation of DNA. The exact length of time that dried tissue can be stored and still yield suitable quantities and quality of genomic DNA should be determined empirically. Other factors, such as stress induced accumulation of phenolic compounds using this approach, may also limit utility and shelf-life of material, and this is likely to vary between species and genotype (SAVOLAINEN *et al.* 1995). Samples can be collected in envelopes and then stored in a sealed container containing silica (such as Silica gel orange with moisture indicator, Sigma-Aldrich catalogue number 13767), with the weight of the silica gel being in at least 10 fold excess compared to plant material (WEISING *et al.* 2005) The PBGL has shown this to be a suitable method for downstream molecular

analysis (figure 1). Silica with moisture colour indicator can be reused by heating the material until the correct colour is observed. However, care should be taken to avoid cross contamination. Using a porous paper envelope, or tea bag to hold tissue limits contact of silica with plant material. Regardless of the method of sample collection and storage, it is advised that the chosen method is thoroughly tested before committing all samples to a specific collection procedure.

Figure 1: Low-cost tissue storage for genomic DNA extraction. Leaf material is collected and placed in envelopes and stored in a box containing silica gel. After 1 day, envelopes containing tissue can be removed and stored under low humidity conditions before DNA extraction (such as in a sealed box or bag with a small amount of desiccant). Tissue grinding can be performed using a standard vortexer and metal ball bearings. An alternative method for tissue drying is to use a desiccation chamber containing 60% glycerol (far right).

3.1: Protocol for plant tissue collection and storage

In this section students will test the silica drying tissue method with wheat or barley leaves collected from the PBGL experimental field.

1) Organize into groups of 3-4, for a total of 8 groups. Each group will be assigned a group letter.
2) Collect tissue envelopes from staff. Label 4 envelopes with your group name and sample number. For example: Group A sample 1, Group A sample 2, and so on.
3) Go to the field and collect leaf tissue. PBGL staff will guide you. Leaf material should be the same length as the collection envelope (approximately 10cm in length, figure 2).

4) Immediately upon collection, place envelopes containing leaf material into a container containing silica gel. Seal the container. The ratio of silica gel to tissue should be no less than 10:1 by weight. Sigma-Aldrich catalogue number 13767 has a moisture indicator. When fully dehydrated and ready for use it is orange, When fully hydrated it turns white (figure 2, right panel). The material can be dehydrated and used many times by heating the material at a low temperature until the colour returns to orange.

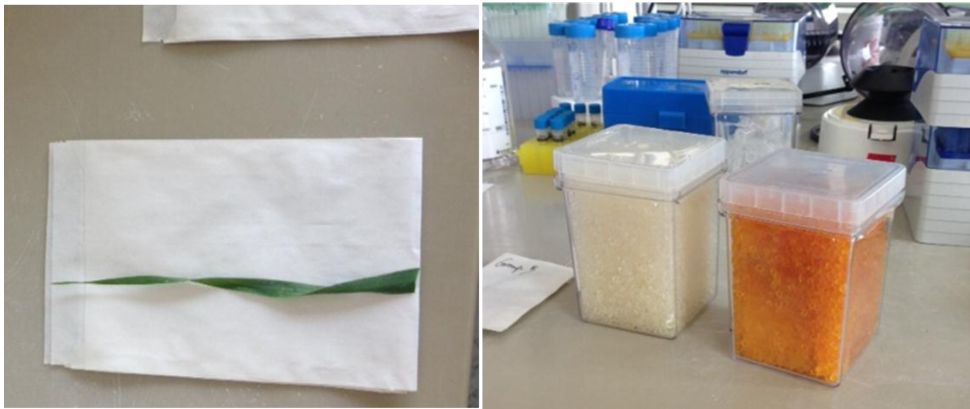

Figure 2: Leaf tissue is collected and placed in an envelope (left panel). The length of the tissue should equal the length of the envelope. Silica gel with colour indicator turns white when fully hydrated (right panel). Only gel with orange colour should be used.

4: Low cost DNA extraction

4.1 OBJECTIVE

The method described in this chapter was developed to avoid toxic organic phase separation utilized in many low-cost DNA extraction protocols such as the CTAB method. The method describes binding of DNA to silica powder under chaotropic conditions, which is a common feature of many commercial kits. The steps of the method involve lysis of plant material, binding of DNA to silica powder, washing and finally elution of DNA from the silica powder. This method has been tested in several plant species and the applicability of such DNA preparations for molecular marker studies in barley is shown.

4.2 MATERIALS (Prepared in advance by the instructors)

Table 1: Chemicals, enzymes and equipment for DNA extraction

MATERIALS AND EQUIPMENT FOR LOW-COST DNA EXTRACTIONS	Suppliers and catalogue numbers (where appropriate)
Celite 545 silica powder (Celite 545-AW reagent grade)	Supelco 20199-U
SDS (Sodium dodecyl sulfate) for mol biol approx 99%	Sigma L-4390-250G
Sodium acetate anhydrous	Sigma S-2889 (MW=82.03g/mol)
NaCl (Sodium chloride)	Sigma S-1314-1KG (MW=58.44g/mol)
RNase A	10 µg/ml
Ethanol	Ethanol absolute for analysis (Merck 1.00983.2500)
Nuclease-free H_2O	Gibco ultrapure distilled water (DNase, RNase-free)
Guanidine thiocyanate	Sigma G9277 (MW=118.2g/mol)
Microcentrifuge tubes (1.5mL, 2.0mL)	Any general laboratory supplier
Micropipettes (1000µL, 200µL, 20µL)	Any general laboratory supplier
Microcentrifuge	Eppendorf Centrifuge 5415D
Optional: Shaker for tubes	Eppendorf Thermomixer comfort for

	1.5mL tubes
MATERIALS FOR GRINDING OF LEAF MATERIAL (depending on grinding method)	
Metal beads (tungsten carbide beads, 3mm)	Qiagen Cat.No. 69997
EVALUATION OF DNA YIELD AND QUALITY	
Agarose gel equipment	Horizontal electrophoresis from any general laboratory supplier

Table 2: Working stocks for DNA extraction

BUFFER	**Recipe**		**Comments**
STOCK SOLUTIONS			
5M NaCl stock solution	MW=58.44g/mol 29.22g / 100mL		Do not use if precipitate forms. Either heat to get fully back into solution or discard and make fresh
3M Sodium acetate (pH 5.2)	MW=82.03g/mol 24.61g / 100mL		Adjust pH value with glacial acetic acid
95% (v/v) Ethanol	95 mL ethanol abs + 5 mL H_2O		Use fresh. Ethanol absorbs water and the % will drop over time.
Tris-EDTA (TE) buffer (10x)	Composition: 100mM Tris-HCl, pH 8.0 10 mM EDTA	For 100mL (10x): 10mL of 1M Tris-HCl stock 2mL of 0.5M EDTA stock	Can make Tris and EDTA from powders. This may be less costly. However, note that the pH of Tris changes with temperature.

LYSIS BUFFER (standard)	0.5% SDS (w/v) in 10x TE 0.5g SDS /100mL	
DNA BINDING BUFFER	6M Potassium Iodide (KI) Alternative: 6M Guanidine thiocynate	**!!!** it takes several hours until dissolved (leave it approx. 4-5 hours)
WASH BUFFER	1mL of 5M NaCl + 99mL of 95% EtOH	**!!!** PREPARE FRESH, because the salt precipitates during storage
DNA ELUTION BUFFER	depending on application (e.g. TE-buffer; Tris-HCl buffer)	

4.3 METHODS

PREPARATION OF SILICA POWDER-DNA BINDING SOLUTION (Performed in Advance by Instructors)

- Fill silica powder (Celite 545 silica) into 50 mL Falcon-tube (to the 2.5mL line = approx. 800mg)
- Add 30 mL dH_2O
- Shake vigorously (vortex and invert)
- Let slurry settle for approx. 15 min
- Remove (pipette off) the liquid
- Repeat 2 times (a total of 3 washes)
- After last washing step: suspend the silica powder in about the same amount of water
  (up to about 5 mL)
- STORE the silica solution at RT until further use (silica : H_2O = 1 : 1)

Before use:

- suspend stored silica solution (silica : H_2O = 1 : 1) by vortexing
- Transfer ~50 µL of silica solution to 2mL tubes (prepare 1 tube per sample)
  !! try to keep the silica suspended during pipetting to ensure an equal distribution

- Add 1mL H_2O (a final wash step)
- Mix by vortexing
- Centrifuge: full speed (13,200) for 10-20 sec
- Pipette off liquid
- Add 700 µL DNA binding buffer (6M Guanidine thiocyanate, or Potassium Iodide)
- Suspend the silica powder in DNA binding buffer
- The silica binding solution is now ready for further use in the protocol (see Methods)

4.3.1: Protocol for low-cost extraction of genomic DNA

1) Prepare an ice bath. Instructors will provide each group with aliquots of all materials needed.
2) Label 2ml tubes containing 3 metal tungsten carbide beads, with your group number and sample number (four total per group).
3) Add dried tissue to the appropriate tube.
4) Tape tubes onto a vortexer and vortex on high setting for 30 seconds or until material is ground in a fine powder (instructors will provide a demonstration).
5). Add 800 µL of Lysis Buffer and 4 µL RNAse A (10 micrograms per millilitre) to each tube.
6) Vortex on high for approximately 2 minutes until powder is fully hydrated and mixed with buffer.
7) Incubate: 10min at room temperature.
8) Add 200 µL 3M Sodium Acetate (pH 5.2). Mix by inversion of tubes and Incubate on ice for 5 min.
9) Centrifuge 13,200 rpm / 5 min / RT (pellet the leaf material)
10) Label the tubes with aliquots of silica binding solution (SBS, 700 microliters) with the group and sample number.
11) Transfer liquid into appropriately labelled SBS containing tubes. DO NOT TRANSFER LEAF MATERIAL.
12) Completely suspend the silica powder by vortexing and inversion of tubes (ca 20 sec)
13) incubate 15 min at RT (on a shaker at 400 rpm and/or invert tubes from time to time)
14) Centrifuge 13,200 rpm / 3 min / RT (pellet the silica)

15) Remove the supernatant with a pipette and discard (the DNA is bound to the silica).
16) Add 500 microliters of freshly prepared wash buffer to each tube.
17) Completely suspend the silica powder by vortexing and inversion of tubes (approx. 20 sec)
18) Centrifuge 13,200 rpm for 3 min at room temperature to pellet the silica. Remove supernatant and keep the pellet.
19) Repeat steps 15-18.
20) Centrifuge pellet for 30 seconds and remove any residual ethanol with a pipette,
21) Open the lid on tubes containing silica pellet and place in fume hood to fully dry pellets for 30 minutes (Optional: This can be done for a longer period on the bench top if a fume hood is not available).
22) Add 200µL TE buffer to each tube to elute the DNA. The DNA is now in the liquid buffer. A buffered solution is preferred over water.
23) Completely suspend the silica powder by vortexing and inversion of tubes (approx. 20 sec)
24) Incubate at room temperature for 5 minutes
24) Centrifuge at 13,200 rpm for 5 min at room temperature to pellet the silica.
25) Label new 1.7 ml tubes with your group name and sample numbers.
26) Collect liquid containing genomic DNA and place into new tubes.
27) Store DNA temporarily at 4°C before checking quality and quantity (NOTE: This would normally be performed immediately, but due to time constraints of the course, this evaluation will be performed in parallel with the evaluation of PCR and mutation discovery.

PLEASE SEE APPENDIX 2 FOR ALTERNATIVE BUFFERS FOR DNA EXTRACTION

5. PCR Amplification

Overview:

In this section, students will perform PCR on genomic DNA to produce a 992 base pair amplicon in the OXI1 gene of Arabidopsis thaliana. Students will be given four genomic DNA samples, 2 which harbor an induced SNP mutation in the gene target and 2 that don't. Following PCR, students will digest an aliquot of the DNA with crude enzyme extract containing single-strand specific nuclease activity. After digestion, samples will be evaluated via agarose gel electrophoresis. The goal is to correctly identify the two mutant samples.

5.1. Protocol for PCR amplification

Materials supplied by the instructors

Genomic DNA from Arabidopsis thaliana (concentration 0.075 ng/µL)
ExTaq PCR buffer (supplied in the ExTaq kit)
dNTPs (supplied in the ExTaq kit)
OXI1L forward primer, Tm 70°C
OXI1R reverse primer, Tm 70°C

Protocol:

1) Place materials provided by instructor on ice
2) Prepare a master mix on ice by combining:
   H_2O 82.5 µL
   10x Ex Taq buffer 15 µL
   2.5 mM dNTP mix 12 µL
   10 µM L primer 1.5 µL
   10 µM R primer 1.5 µL
   TaKaRa HS taq (5 U/µL)* 0.38 µL (the instructor will deliver Taq polymerase when you have mixed the other components)
3) Label 4 PCR tubes with your group name and sample number.
4) Combine 7.5 µL DNA with 22.5 µLPCR mix.

5) Incubate in thermal cycler using the program PCRTM70 that was previously prepared by instructors:

95°C for 2 min; loop 1 for 8 cycles (94°C for 20 s, 73°C for 30 s, reducetemperature 1°C per cycle, ramp to 72°C at 0.5C/s, 72°C for 1 min); loop 2 for 45cycles (94°C for 20 s, 65°C for 30 s, ramp to 72°C at 0.5°C/s, 72°C for 1 min); 72°C for 5 min; 99°C for 10 min; loop 3 for 70 cycles (70°C for 20 s, reduce temperature 0.3°C per cycle); hold at 8°C.

This program takes approximately 4 hours, and the thermal cycler is programmed to store samples at 8°C overnight.Once you have the PCR running, prepare an agaorse gel for Day 3. Once poured and solidified, the gel can be stored at 4°C overnight.

5.2 Agarose gel preparation (DEMONSTRATED BY INSTRUCTOR). Four groups will share a single gel.

Preparation: 300mls 1.5% agarose with a final concentration of 0.5 μg/ml Ethidium Bromide (CAUTION: Ethidium Bromide is toxic, wear gloves and goggles).

1) Combine 4.5 g agarose with 0.5x TBE running buffer. Microwave until agarose is completley melted.
2) Allow to cool to about 60°C (you can hold the bottle in your hands for 3-5 seconds before it is too hot to hold).
3) Add 15 μL of 10 μg/μL ethidium bromide stock. Pour the gel, insert the combs and allow to solidify.
4) Once the gel has hardened, wrap in plastic wrap and store at 4°C.

6: Enzymatic mismatch cleavage and agarose gel evaluation of all student samples

6.1 Enzymatic mismatch cleavage

After PCR amplification, denaturation and annealing of PCR products allows DNA strands with small sequence differences to hybridize together. The result is heteroduplexed molecules where the othewise doubled stranded DNA is single-stranded where mutations exist. These are the substrates for cleavage by single strand specific nucleases such as CEL I, crude Celery Juice Extract (CJE) containing CEL I, and crude weed enzyme extract. See appendix 3 for a protocol for a large-scale prep of nuclease from weeds collected in the wild. These methods can be scaled down for table-top microfuges using protein retention columns. If interested, please contact PBGL for this protocol.

Materials provided by the instructor (place on ice bath):

10x CELI buffer
Crude enzyme extract
1kb DNA ladder
EDTA

1) Prepare the following mix on ice (calculated for 5 samples):
   81.5 µl water
   15 µl 10x CEL I TILLING buffer
   3.5 µl CJE nuclease
2) Label 4 new PCR tubes with your group name, sample number and the letter x (to differentiate this from the original PCR product.
3) Combine 20 µL of PCR product with 20 µL of enzyme master mix. Mix the components.
4) Incubate at 45°C for 15 minutes in a thermal cycler (pre-programed by the instructors).
5) Place the reactions on ice, and stop the reaction by adding 10 µL of 0.25M EDTA per sample. Mix and centrifuge.

To make 10x CEL I buffer, mix:

5 ml 1M $MgSO_4$ 100 µl 10% Triton X-100

5 ml 1M Hepes (pH 7.4) 5 µl 20 mg/ml bovine serum albumen

2.5 ml 2M KCl 37.5 ml water

6.2 Loading samples on agarose gels:

In this section of the course, students will evaluate all of the materials they have prepared: genomic DNA, PCR product and PCR product digested with nuclease. All 12 samples and two ladder lanes will be run on the gel.

4 groups will share a single gel.

Group	Gel #	Comb	Left or right half
A	1	Top	Left
B	1	Top	Right
C	1	Bottom	Left
D	1	Bottom	Right
E	2	Top	Left
F	2	Top	Left
G	2	Bottom	Right
H	2	Bottom	Right

Prepare the following mixes

Tube/lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sample	1Kb ladder	Genomic 1 (G1)	G 2	G 3	G 4	PCR 1	PCR 2	PCR 3	PCR 4	Digest 1 (D1)	D 2	D 3	D 4	1 kB ladder
Volume (microliter)	1	5	5	5	5	10	10	10	10	10	10	10	10	1
Loading dye	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Water	9	5	5	5	5	X	X	X	X	X	X	X	X	9

Run the gel for 45 minutes at 120 volts.

6.3: Gel photography and data analysis

Students should evaluate the photograph of their gel and draw conclusions about their data. Please check the following boxes as you feel appropriate and then show your gel data and show this table to the instructors. The instructors will project gel data and discuss in a group

Genomic DNA	High quality	Good yield	Degradation	Low yield	RNA contamination
1					
2					
3					
4					

PCR product	Specific amplifcation	Non-specific amplification	High yield	Low yield
1				
2				
3				
4				

Digested DNA	Full-length PCR product	No visible PCR product	Cleavage products (give molecular weights)	No Cleavage products
1				
2				
3				
4				

7: Example data

7.1 Quality of genomic DNA obtained by silica powder-based DNA extraction method

While spectroscopic methods such as the Nanodrop provide a quick and accurate measure of DNA concentration and protein contamination, and fluorometric methods such as the Qbit provide high sensitivity, it is suggested that when optimizing the DNA extraction protocol, samples are also run on a traditional agarose gel. This allows a quick snapshot of the concentration of DNA, extent of RNA carryover, and also provides an estimation of the extent of DNA degradation, something which cannot be easily estimated from the other two methods. Further, chaotropic salts can reduce the accuracy of spectrophotometric methods. Reducing sample degradation may be a key optimization step for some species. Alternative buffers can be employed to reduce sample degradation and reduce the co-purification of secondary metabolites that can inhibit downstream molecular assays.

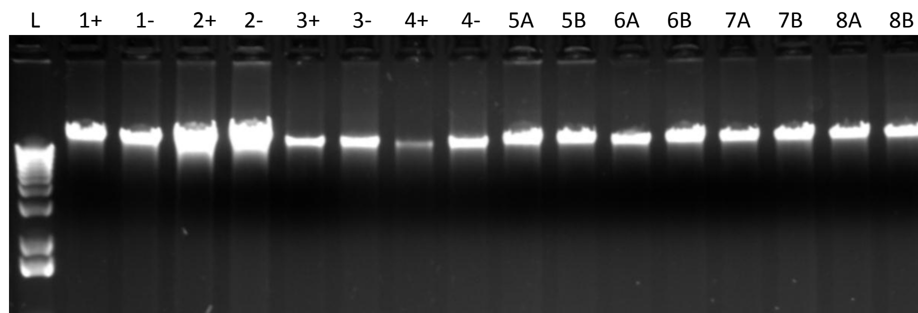

Figure 3. Quality of barley genomic DNA extractions using silica powder and different combinations of self-made (low-cost) buffers and buffers provided by Qiagen DNeasy kit. 8 μL of each genomic DNA extraction were separated on a 0.7% agarose gel.

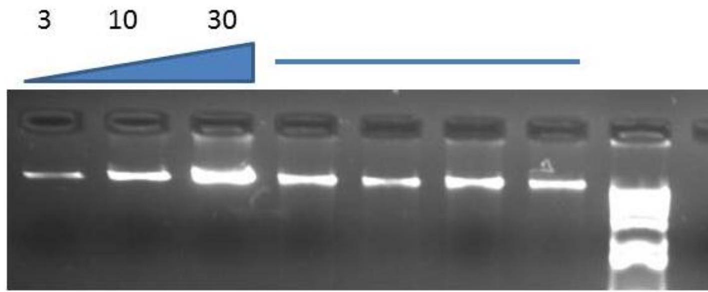

Figure 4: Example data produced by participants of the June 3-14, 2013 training course on "Plant Mutation Breeding: Mutation Induction, Mutation Detection, and Pre-Breeding". Triangle denotes lanes with lambda concentration standard (concentrations in nanograms/microliter listed above). The blue bar denotes student samples. 100% of student samples were of high quality and sufficient quantity for molecular assays, even though some students had never before worked in the field of molecular biology.

7.2 Example of PCR products using TILLING primers with source genomic DNA from Qiagen kits and home-made PBGL silica method.

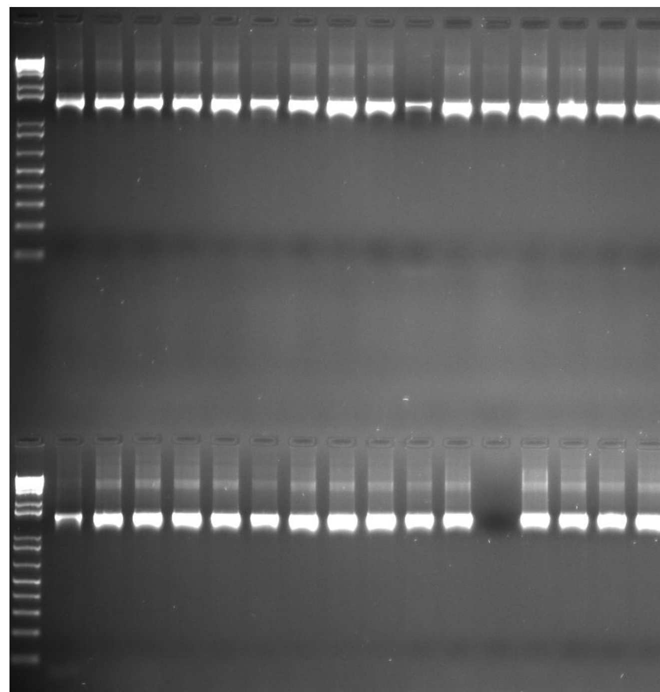

Figure 5. TILLING-PCR products amplified from genomic DNA extractions of barley (obtained by silica-based, low-cost DNA isolation method using different combinations of

self-made buffers and buffers provided by Qiagen DNeasy kit). An aliquot of 5µL of each PCR reaction was separated on a 1.5% agarose gel. Different primers were used for the top and bottom half of the gel (barley nb2-rdg2a and nbs3-rdg2a respectively), and the first 8 samples were prepared with Qiagen DNA preps and the last half following the PBGL home-made silica protocol.

7.3 Example of low-cost agarose gel based TILLING assays for the discovery of induced point mutations.

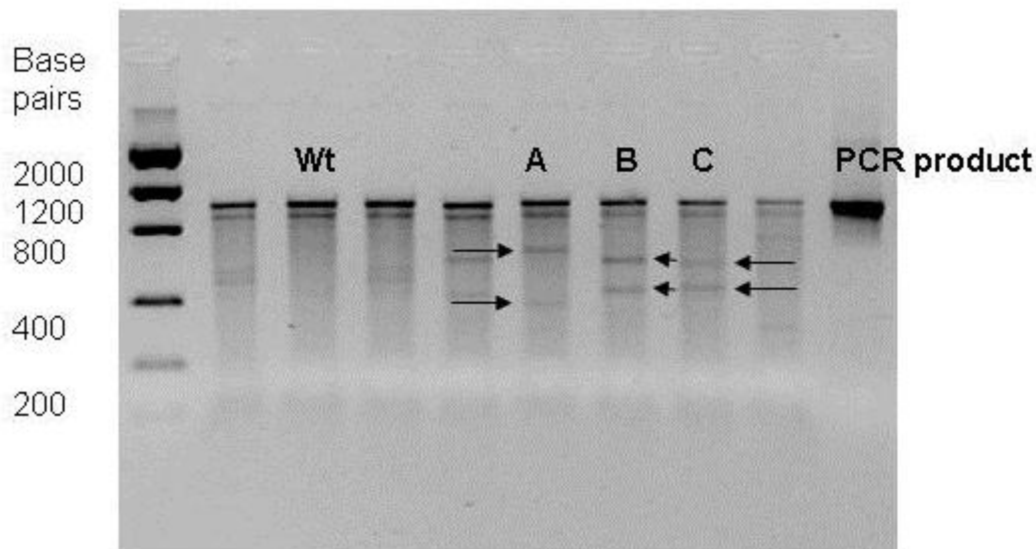

Figure 6 Gel image of mutation discovery using crude celery juice extract for enzymatic mismatch cleavage followed by visualization by agarose gel electrophoresis. Test samples included in this kit are marked above image as is an example of undigested PCR product. Molecular weights of ladder bands are listed. Bands representing cleavage products at the site of mutation are marked by arrows. Two bands are produced upon double strand cleavage at the site of a mutation. The sizes of the cleaved fragments sums to the size of the full length PCR product. This image was produced at the 2009 FAO/IAEA International Training Course on Novel Biotechnologies for Enhancing Mutation Induction Efficiency by Mr. Saad Alzahrani of Saudi Arabia, and Mr Azhar Bin Mohamad of Malaysia.

8: Conclusions

The methods described here provide rapid and low-cost alternatives for sample preparation and genomic DNA extraction. When evaluating methods, it is important to remember that protocol adaptations will potentially be necessary to compensate for sample differences (species and genotype), and also environmental conditions in the laboratory and quality of the water and chemicals used. Saving a few euros in DNA preparation is no good if the samples are unsuitable for downstream applications like PCR, or they have a very short shelf-life. With the appropriate validation of sample quality and longevity, the protocol described here can provide sufficient DNA for a variety of molecular applications such as marker studies and TILLING, at a cost of only 10% per sample compared to commercial kits.

9: References

CHASE, M. W., and H. H. HILLS, 1991 Silica gel:an ideal material for field preservation of leaf samples for DNA studies. Taxon **40:** 215-220.

COLBERT, T., B. J. TILL, R. TOMPA, S. REYNOLDS, M. N. STEINE *et al.*, 2001 High-throughput screening for induced point mutations. Plant Physiol **126:** 480-484.

COMAI, L., K. YOUNG, B. J. TILL, S. H. REYNOLDS, E. A. GREENE *et al.*, 2004 Efficient discovery of DNA polymorphisms in natural populations by Ecotilling. Plant J **37:** 778-786.

DAHM, R., 2005 Friedrich Miescher and the discovery of DNA. Dev Biol **278:** 274-288.

DELLAPORTA, S. L., J. WOOD and J. B. HICKS, 1983 A plant DNA minipreparation: version II. . Plant Mol. Biol. Rep. **1:** 19-21.

JANKOWICZ-CIESLAK, J., O. A. HUYNH, S. BADO, M. MATIJEVIC and B. J. TILL, 2011 Reverse-genetics by TILLING expands through the plant kingdom. Emirates Journal of Food and Agriculture **23:** 290-300.

JANKOWICZ-CIESLAK, J., O. A. HUYNH, M. BROZYNSKA, J. NAKITANDWE and B. J. TILL, 2012 Induction, rapid fixation and retention of mutations in vegetatively propagated banana. Plant Biotechnology Journal **10:** 1056-1066.

LISTON, A., L. H. RIESEBERG, R. P. ADAMS, N. DO and G. ZHU, 1990 A method for collecting dried plant specimines for DNA and isozyme analyses, and the results of a field experiment in Xinjiang, China. Ann. Missouri Bot. Gard. **77:** 859-863.

MCCALLUM, C. M., L. COMAI, E. A. GREENE and S. HENIKOFF, 2000 Targeted screening for induced mutations. Nat Biotechnol **18:** 455-457.

MURRAY, M. G., and W. F. THOMPSON, 1980 Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res **8:** 4321-4325.

SAVOLAINEN, V., P. CUENOUD, R. SPICHIGER, M. D. MARTINEZ, M. CREVECOEUR *et al.*, 1995 The use of herbarium specimens in DNA phylogenetics: evaluation and improvement. Plant Syst. Evol. **197:** 87-98.

TILL, B. J., C. BURTNER, L. COMAI and S. HENIKOFF, 2004a Mismatch cleavage by single-strand specific nucleases. Nucleic Acids Res **32:** 2632-2641.

TILL, B. J., J. JANKOWICZ-CIESLAK, L. SAGI, O. A. HUYNH, H. UTSUSHI *et al.*, 2010 Discovery of nucleotide polymorphisms in the Musa gene pool by Ecotilling. Theor Appl Genet **121:** 1381-1389.

TILL, B. J., S. H. REYNOLDS, E. A. GREENE, C. A. CODOMO, L. C. ENNS *et al.*, 2003 Large-scale discovery of induced point mutations with high-throughput TILLING. Genome Research **13:** 524-530.

TILL, B. J., S. H. REYNOLDS, C. WEIL, N. SPRINGER, C. BURTNER *et al.*, 2004b Discovery of induced point mutations in maize genes by TILLING. BMC Plant Biol **4:** 12.

WEISING, K., H. NYBOM, K. WOLFF and G. KAHL, 2005 *DNA Fingerprinting in Plants: Principles, Methods and Appiclations.* CRC Press, Boca Raton.

10. Appendices

Appendix 1: Preparation of a home-made chemical spill kit.

All laboratories should contain a spill kit for chemical spills. While commercially available kits are available, self-prepared kits can be made at a fraction of the cost. Key materials and their use are found in table 7. The kit should be designed to handle a spill from the largest volume of chemical you have in the laboratory.

Table 7: Components of a chemical spill kit and their uses.

Component	Use
5 gallon plastic or rubber bucket with lid clearly labelled "Chemical Spill Kit", with emergency telephone numbers printed clearly on the lid and side of the bucket.	This bucket contains all the materials of the spill kit, and should be located near the laboratory doorway to allow someone to access it after they have left the spill area.
Goggles	For eye protection while cleaning spill
Chemical resistant gloves	For hand protection when dealing with spills
Absorbent materials (Cat litter, vermiculite, activated charcoal or sawdust)	This material is placed on the liquid spills to contain the liquid for easy removal.
Small broom and plastic dustpan	For removal of dry spills, and absorbed materials. It is important that the dustpan or scoop be plastic and metal materials can spark and cause fire/explosions.
Sturdy plastic bags	To contain materials
Baking soda (sodium bicarbonate), in a plastic bag marked "for liquid acid spills".	For neutralization of small acid spills
Acetic acid powder in a plastic bag marked "for liquid base spills"	For neutralization of small base spills.

Note that material collected after a spill should not go into the normal waste but be disposed of in the appropriate manner according to the local guidelines for hazardous waste.

Appendix 2: Alternative buffers for DNA extraction.

The main areas for optimization of DNA extraction methods includes increasing sample yield, reducing co-purification of unwanted components such as polysaccharides, and polyphenols, and reducing sample degradation. To a large extent, providing the starting tissues are of good quality, all three areas can be influenced by the sample lysis procedure. The table below lists four lysis buffers used in experiments following the protocol in chapter 5 to optimize isolation of DNA from grapevine and sorghum. A more thorough compilation of buffer components and additives to enhance DNA isolation in the presence of secondary compounds can be found in (WEISING *et al.* 2005).

Table 5: Alternative lysis buffers for DNA extraction

LYSIS BUFFER – LB1	Composition 0.5% SDS (w/v) in 10x TE	For 100mL 0.5g SDS /100mL in 10x TE
LYSIS BUFFER – LB2	Composition - 0.5% SDS (w/v) - 0.5M NaCl - 3% PVP (w/v) in 10x TE	For 100mL - 0.5g SDS /100mL - 10mL of 5M NaCl - 3g / 100mL in 10x TE
LYSIS BUFFER – LB3	Composition - 0.5% SDS (w/v) - 0.5M NaCl - 3% PVP (w/v) - 1% sodium sulphite in 10x TE	For 100mL - 0.5g SDS /100mL - 10mL of 5M NaCl - 3g / 100mL - 1g / 100mL in 10x TE
LYSIS BUFFER – LB4	Composition - 0.5% SDS (w/v) - 0.5M NaCl - 3% PVP (w/v) - 1% sodium sulphite - 2% N-lauryl-sarcosyl sodium salt in 10x TE	For 100mL - 0.5g SDS /100mL - 10mL of 5M NaCl - 3g / 100mL - 1g / 100mL - 2g / 100mL in 10x TE

Results:

DNA CONCENTRATIONS AND YIELDS

Table 6. Sorghum and grapevine genomic DNA extractions using the silica powder method with 4 different lysis buffers LB1, LB2, LB3 or LB4

	SORGHUM								**GRAPEVINE**							
Sample	S1 a	S1 b	S2 a	S2 b	S3 a	S3 b	S4 a	S4 b	G1 a	G1 b	G2 a	G2 b	G3 a	G3 b	G4 a	G4 b
Lysis buffer	**Lysis buffer LB 1**		**Lysis buffer LB 2**		**Lysis buffer LB 3**		**Lysis buffer LB 4**		**Lysis buffer LB 1**		**Lysis buffer LB 2**		**Lysis buffer LB 3**		**Lysis buffer LB 4**	
Incubation temperature (for lysis)	RT	65^0 C	RT	65^0 C	RT	65^0 C	RT	65^0 C	RT	65^0 C	RT	65^0 C	RT	65^0 C	RT	65^0 C
DNA concentration (ng/μL)	**11**	**15**	**2**	**3**	**6**	**5**	**7**	**7**	**22**	**32**	**7**	**3**	**11**	**6**	**4**	**9**
Total yield (μg)	2.0	2.6	0.4	0.6	1.0	0.9	1.3	1.3	3.9	5.7	1.3	0.6	2.1	1.1	0.6	1.5

QUALITY OF GENOMIC DNA

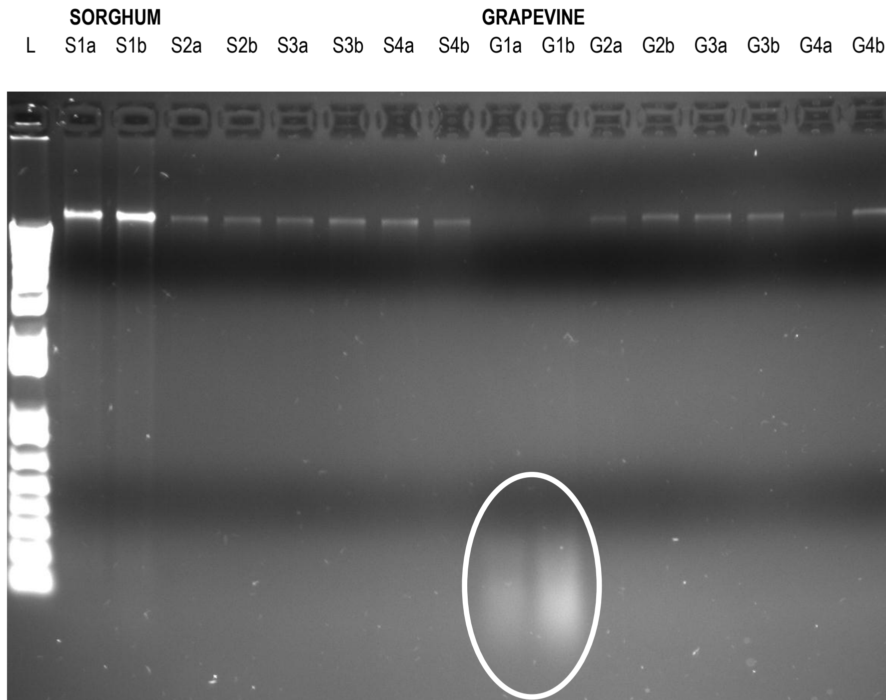

Figure 7. Quality of genomic DNA extracted from Sorghum and grapevine using the silica powder method with four different lysis buffers. 8 µL of each genomic DNA preparation were separated on a 0.7% agarose gel. S and G indicate sorghum or grapevine; Numbers 1-4 represent lysis buffers LB1 -4 respectively. a=incubation in lysis buffer for 10 min at room temperature, b=incubation in lysis buffer for 10 min at 65^{0}C, L: size standard (1 kB Plus DNA ladder - Invitrogen)

This experiment shows that high yield and high quality genomic DNA can be recovered from Sorghum using Lysis buffers 1 This buffer , however, are not suitable for grapevine DNA extraction and alternative buffers are required to recover high molecular weight DNA, albeit at a lower concentration than can be achieved from Sorghum samples. This suggests further parameter changes can be made to increase yields.

Appendix 3: Alternative enzymology for mismatch cleavage for TILLING and Ecotilling: extraction of enzymes from weedy plants.

Version 1.4, Plant Breeding and Genetics Laboratory, February 8th, 2013. Prepared by Bernhard Hofinger and Bradley Till

1. OBJECTIVE

A crude celery extract containing the single-strand-specific nuclease CELI has been widely used in TILLING and Ecotilling projects around the world. Yet, celery is hard to come by in some Member States. Based on previous studies and bioinformatic analysis suggestion homologies exist to CELI in all plants. Therefore, we developed a protocol for extraction of active enzyme from plants common across the world: weeds. We isolated weed plants from the grassland around the Seibersdorf laboratories and isolated a crude enzyme extract (in parallel to the enzyme extracts from celery). Since, there was no or only very low mismatch digestion activity in the crude extract, we applied a centrifuge-based filter method to concentrate the enzyme extract.

2. MATERIALS

MATERIALS / BUFFERS FOR ENZYME EXTRACTIONS	Notes
hand-held mixer (or juicer)	From any supplier
STOCK: 100mM PMSF (stock in isopropanol)	To prepare an aqueous solution of 100µM PMSF (for buffers A and B), add 1 ml 0.1M PMSF per liter of solution immediately before use.
STOCK: 1M Tris-HCl, pH 7.7.	
Buffer A: 0.1 M Tris-HCl, pH 7.7, 100 µM PMSF.	
Buffer B: 0.1 M Tris-HCl, pH7.7, 0.5 M KCl, 100 µM PMSF.	
Dialysis tubing with a 10,000 Dalton	e.g. Spectra/PorR Membrane MWCO:

molecular weight cut off (MWCO)	10,000, Spectrum Laboratories, Inc.
$(NH_4)_2SO4$ (Ammonium sulphate)	
Sorvall Centrifuge	Or equivalent centrifuge/rotor combination to achieve needed gravitational force
MATERIALS FOR CONCENTRATION OF ENZYME EXTRACTS	
Amicon Ultra Centrifugal Filters (0.5mL, 10K)	Millipore Amicon Ref.No. UFC501024 24Pk
Refrigerated (4°C) Microcentrifuge	e.g. Eppendorf 5415R
TILLING-PCR	
Thermocycler	e.g. Biorad C1000 Thermal cycler
PCR tubes	Life Science No 781340
TaKaRa Ex Taq™ Polymerase (5U/ul)	TaKaRa
10X Ex Taq™ Reaction Buffer	TaKaRa
dNTP Mixture (2.5mM of each dNTP)	TaKaRa
Agarose gel equipment	

3. METHODS

3.1 Enzyme extraction

1. Collect approximately 200 grams of mixed monocot and dicot weedy plants were collected that were growing on the periphery of our sorghum field.

2. Wash material 3x in water and then ground using a hand-held mixer and by adding about 300 mls of water to facilitate tissue disruption (or optional in a juicer)

3. Add 1M Tris-HCl (pH7.7) and 100mM PMSF to a final concentration of Buffer A (=0.1M Tris-HCl and 100µM PMSF) (NOTE: Stocks and water should be kept at 4°C, perform subsequent steps at 4°C)

4. Centrifuge for 20 min at 2600 x g in Sorvall GSA rotor to pellet debris. Save supernatant.

5. Bring the supernatant to 25% ammonium sulphate (add 144 g per litre of solution). Mix gently at 4°C (cold room) for 30 min.

6. Centrifuge for 40 min at 4°C at ~14,000 x g in sorvall GSA rotor (~9000 rpm). Discard the pellet.

7. Bring the supernatant to 80% ammonium sulphate (add 390 g per liter of solution). Mix gently at 4°C for 30 min.

8. Centrifuge for 1.5 hours at 4°C at ~14,000 x g in sorvall GSA rotor. SAVE the pellet. Discard the supernatant (be careful in decanting the supernatant!) The pellet can be stored at –80°C for at least two weeks.

9. OPTIONAL: Pellets can be frozen at -80°C for months.

10. Resuspend the pellets in ~ 1/10 the starting volume with Buffer B (Frozen pellets of the weed juice extract were suspended in 15mL Buffer B and pellets of the celery juice extract in 10 mL Buffer B). Ensure the pellet is thoroughly resuspended.

11. Dialyze against Buffer B at 4°C (2 Liters per 10mls of resuspended solution). Use e.g. Spectra por 7 MWCO 10000 tubing. (NOTE: Soak the dialysis tubing in nanopure water for 30 min. before use.)

12. Dialyze for 1 hour against Buffer B at 4°C

13. Repeat for a total of 4 dialysis steps with a minimum of 4 hours dialysys. (NOTE: Longer dialysis is better, it is often convenient to perform the third dialysis overnight).

14. Remove liquid from dialysis tubing. It is convenient to store ~75% of the liquid in a single tube at -80°C and the remainder in small aliquot for testing.This protein mixture does not require storage in glycerol and remains stable through multiple freeze-thaw cycles, however, limiting freeze thaw cycles to 5 limits the chance of reduced enzyme activity

15. Perform activity test (step 3.3, or proceed immediately to enzyme concentration, step 3.2)

Figure 8. Mixture of different plant species (weedy plants) from the grassland around the Seibersdorf laboratories used for the isolation of an enzyme extract for mismatch cleavage.

3.2 CONCENTRATION OF ENZYME EXTRACTS of weed and celery enzyme extracts using Amicon Ultra 10K centrifugal filter devices (for 0.5mL starting volume; in 1.5-mL tubes)

1. Perform with 600µL of protein extract after dialysis
2. Clear extract by centrifugation at 30 min / 10 000 x g / 4°C (to pellet plant material) in refrigerated microcentrifuge
3. Transfer 500 µL of the (cleared) supernatant to a filter device (keep the rest of the supernatant as control “before concentration”).
4. Centrifuge the filter device with a collection tube inserted per manufacturers instructions for 30 min / 14 000 x g / 4°C
5. Remove filter device, invert and place in new collection tube.
6. Centrifuge for 2 min / 1 000 x g / 4°C
7. Measure the recovered volume. This is your concentrated protein. Calculate the concentration factor with the following formula: Starting volume/Final folume = concentration factor

a. TEST OF MISMATCH CLEAVAGE ACTIVITY

1. Produce TILLING-PCR products for mismatch cleavage tests with the concentrated enzyme extracts. The exmple below is for barley.

GENES/PRIMER: nb2-rdg2a (1500bp-PCR product)

nb2-rdg2a_F2	TCCACTACCCGAAAGGCACTCAGCTAC
nb2-rdg2a_R2	GCAATGCAATGCTCTTACTGACGCAAA

TILLING PCR REACTIONS (TaKaRa ExTaq enzyme): total volume: 25µL

10x ExTaq buffer (TaKaRa)	2.5 µL
dNTP mix (2.5 mM)	2.0 µL
Primer forward (10 µM)	0.3 µL
Primer reverse (10 µM)	0.3 µL
TaKaRa Taq (5U /µl)	0.1 µL
Barley genomic DNA (5 ng/µL)	5.0 µL
H_2O (to 25 µL)	14.8 µL

TILLING PCR cycling program for TILLING ("PCRTM70")

95°C for 2 min;
loop 1 for 8 cycles (94°C for 20 s, 73°C for 30 s, reduce temperature 1°C per
cycle, ramp to 72°C at 0.5°C/s, 72°C for 1 min);
loop 2 for 45 cycles (94°C for 20 s, 65°C for
30 s, ramp to 72°C at 0.5°C/s, 72°C for 1 min);
72°C for 5 min;
99°C for 10 min;
loop 3 for 70 cycles (70°C for 20 s, reduce temperature 0.3°C per cycle);
hold at 8°C.

2. Mix 10µL of PCR product with 10µL weed digestion mix to a volume of 20µL

3. Incubate at 45°C for 15 min

4. Add 2.5µL of 0.5M EDTA (pH 8.0) – to stop reaction

4. Load a 10µL aliquot on an agarose gel

4. Example results

Concentrations of protein extracts:

Table 1. Calculations of concentration factors after centrifugation with Amicon Ultra 10K – Starting volume: 500 µL ("Before" centrifugation = considered as 1x concentrated)		
	Recovered volume	**Concentration factor** **(calculated from 500 µL starting volume)**
Weed	~42 µL	**11.9x**
Cell	~33 µL	**15.2x**

Mismatch digestions using celery and weed enzyme extracts:

Table 2. Set-up of mismatch digestions using celery and weed enzyme before and after centrifugation with Amicon Ultra 10K. The enzyme concentration in the extracts were calculated using the calculated concentration factors from Table 1.				
	1 - BEFORE	**2 - after**	**3 - after**	**4 – after**
Enzyme	3.5 µL (1x)	0.5 µL	3 µL	6 µL
Cell buffer	1.5 µL	1.5 µL	1.5 µL	1.5 µL
H_2O	5 µL	8.0 µL	5.5 µL	2.5 µL
Tot.Volume	10 µL	10 µL	10 µL	10 µL
Celery enzyme concentration in relation to extract before centrifugation (3.5µL – before = 1x)	**1x**	7.6 µL **2.2x**	45.6 µL **13.0x**	91.2 µL **26.1x**
Weed enzyme concentration in relation to extract before centrifugation (3.5µL - before = 1x)	**1x**	5.95 µL **1.7x**	35.7 µL **10.2x**	71.4 µL **20.4x**

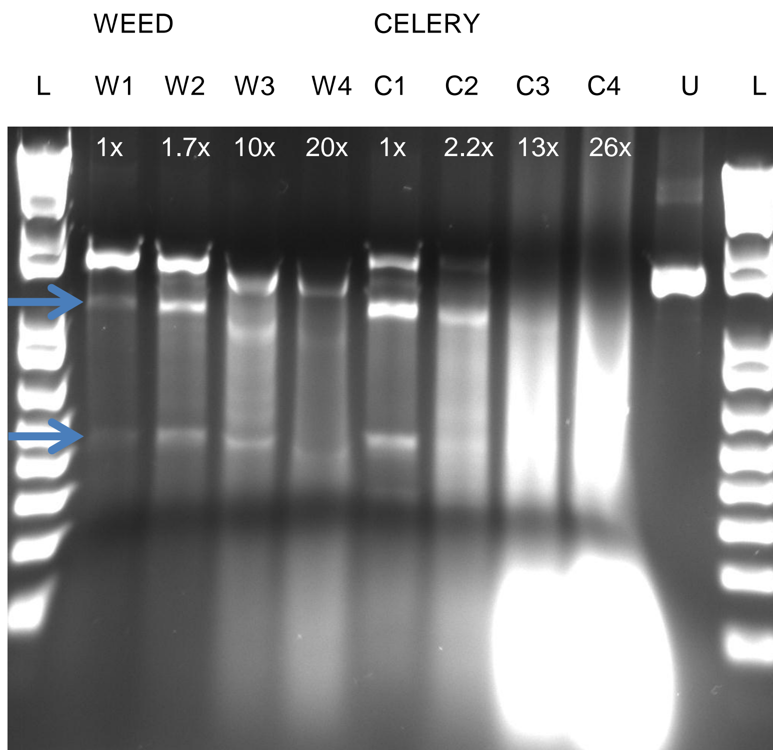

Figure 9. Mismatch cleavage with celery and weed enzyme extracts. TILLING-PCR products of the target gene nb2-rdg2a (1500bp-PCR product) were produced from genomic DNA of barley. The PCR products were digested with weed and celery enzyme extracts before and after concentration by centrifugation with Amicon Ultra 10K. 10 µL of the digested PCR products were separated on a 1.5% agarose gel. Position of SNPs are marked with blue arrows. Concentrations of Weed (W) and Celery (c) extracts are listed above the lanes. A 1kb ladder is loaded on either side of the samples.

5. CONCLUSIONS

Crude enzyme extracts of weeds show a similar activity to that of celery extract for the cleavge of single nucleotide polymorphisms. The per unit activity, however, was lower than than for CEL I, likley owing to the co-precipitation of other plant proteins in weeds, presumably including a larger amount of RUBISCO. This limitation can be overcome through the use of a simple centrifugation based protein concentration step. 150 mls of weed extract produces enough enzyme for approximately 2000 reactions with this protocol.