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Meeting Report

TILLING and Associated Technologies

As part of a collaboration between the John Innes Centre (JIC), Norwich, UK and the Centre for Signal Transduction and Metabolomics (C-STM) at the Institute of Botany (IOB), the Chinese Academy of Sciences, Beijing, a workshop on 'TILLING and Associated Technologies' was held at the IOB on 15–16 April 2010. Through sponsorship from the UK Biotechnology and Biological Sciences Research Council (BBSRC), Resarch Councils UK, China Office, and the Transgenic Research Project of China, experts were drawn from the USA, Europe and China to present their latest information on technology development for targeted single nucleotide polymorphism (SNP) detection to over 60 registered participants. The main focus of the workshop was the non-genetic modification (non-GM) strategy of TILLING (Targeting Induced Local Lesions in Genomes) and ecotype TILLING (eco-TILLING), which can be used both for gene function analysis and for crop improvement.

The keynote speaker, Brad Till (International Atomic Energy Agency/Food and Agriculture Organisation (IAEA/FAO), Vienna), formerly manager of the Seattle TILLING Project (STP) and part of the group that developed the original methodologies, introduced the history of the field and gave a summary of the IAEA's work with developing countries on TILLING of vegetatively propagated crops such as banana and cassava. They are concentrating on traits for stress and disease resistance and have made a considerable advance using chemical mutagenesis of *in vitro* cultured plant materials followed by micropropagation. Mutations had been shown to be stably transmitted across seven rounds of micropropagation by meristem division in banana. Stocks of mutant plants are held in culture for distribution in a similar way to seed stocks maintained for seed bearing species.

The main focus of the first day was cereal crops and these presentations were kicked off by the recent ground-breaking work of the University of California (UC) Davis TILLING Core Service Facility (USA). The head of this group, Luca Comai was originally part of the STP. Helen Tsai, the manager of the TILLING Core in Davis gave a comprehensive outline of their development of a TILLING-by-Sequencing platform based on Illumina sequencing that they will be using for *Arabidopsis*, *Camelina*, tomato, rice and wheat. She explained how they used 3-D pooling and stacking up to 40 genes at a time. For next-generation (NextGen) sequencing applications, DNA quantification (they currently use Sybrgreen) for pooling was paramount, more so than for gel sequencers. Gel purification of polymerase chain reaction (PCR) products was also very important. An example for their platform, TILLING of wheat *VRN3* was presented to show that the methodology was robust. Currently, it is their intention to develop bar-coding primers to help identify individual plants, to examine the possibility to use non-specific primers in polyploids to simultaneously amplify homoeologues, and to look at methods for eco-TILLING. Tailor-made software has been developed for analyzing the sequence data and it will be released shortly for community use.

Lei Shi from Huazhong Agricultural University, Wuhan discussed their recent data on eco-TILLING by whole genome re-sequencing of *Brassica napus* (rapeseed). They have identified several different haplotypes in both *B. napus* and *Brassica Oleraceae,* which have allowed them to pinpoint the ancestry of rapeseed varieties. They have examined the genetic basis of erucic acid content by mining over 101 rapeseed lines and concluded that there is no new variation available in ecotypes, so TILLING may be very important in generating novel variation.

Cristobal Uauy (John Innes Centre, Norwich, UK) discussed the problems of and solutions for primer design for TILLING in polyploid species. Wheat can bear a large number of mutations per genome because of its polyploid nature so only small populations are required to cover the mutational landscape. He has been using both tetraploid and hexaploid wheats, the latter being better for gene functional analysis as a single generation of crosses followed by selection of homozygous double mutants in the F_2 populations is sufficient to generate null mutants. Since primer design is currently the most labor intensive part of wheat TILLING, he outlined their simple polyacrylamide gel electrophoresis (PAGE) based detection method that eliminates the need for fluorescently labeled primers, which can basically be used in any laboratory. This is an especially attractive approach for complex genomes where high mutation densities have been achieved.

Tetraploid wheats were also the main subject of Martin Parry's (Rothamsted Research, UK) presentation. He outlined

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their TILLING platform based around high resolution melting technology, where the gibberellin-metabolizing enzyme GA20 oxidase gene was used as a target in their studies. He also gave details of their successful screens in durum wheat for drought and salt tolerance, from which material had been passed to the International Center for Agricultural Research in the Dry Areas (ICARDA) for incorporation into breeding programs.

Aimin Zhang from The Institute of Genetics and Developmental Biology (IGDB), Beijing described their eco-TILLING work in Chinese wheats to discover novel stature varieties by targeting hormone genes, especially *pin* and *rht*. They have also looked for variants specifically in the local cv. Xiaoyan 54 for *pin* and *idx14* genes.

The current BBSRC UK-China collaboration was established to provide a rice TILLING platform at IOB. Chun-Ming Liu, the principal investigator involved at IOB, outlined the rice materials developed for this platform. They have used both ethyl methane sulphonate (EMS) and sodium azide to mutagenize rice, where azide was found to be particularly effective. Altogether they have approximately 20000 mutagenized M_2 lines at C-STM and have carried out both forward and reverse screens. Their interest is in seed development and especially the aleurone layer to increase the nutritional value of the grain.

Hai-chun Jing (IOB) described their comparative analyses of maize and sorghum. Sorghum is the "camel of the crops", being tolerant to drought and saline stresses. Sorghum has a small genome, high biomass, but low grain yield, in contrast to maize, which has a high grain yield. They have used NextGen sequencing to cover three sorghum and three maize genomes for comparative purposes and to date have mutagenized populations of approximately 1500 lines each of the three maize lines and 5000 for sorghum cv. 'Keller'. In sorghum they have found much variation for a potassium transporter believed to be involved in the salt tolerance trait. Other targets include grain development, and sugar and starch metabolism.

On the second day, the focus changed to other species. Trevor Wang (JIC), the UK principal investigator for the BBSRC project, described the materials that they have assembled for their *RevGen*UK TILLING service (revgenuk@bbsrc.ac.uk). This is a cost-recovery service delivering mutants currently for the model legumes, *Lotus japonicus*, *Medicago truncatula* and for *Brassica rapa* to researchers across the globe. He also outlined their plans to expand the service to other models and crops. From their initial studies on lotus, which was the first platform outside Seattle to be established, they have shown that there is a bias for glycine replacements in functionally defective alleles. He also provided evidence that lotus has three genetically effective cells in the meristem germline in contrast to the one of arabidopsis. This means in practice that heterozygous mutants are recovered much more frequently than homozygotes (10:1).

Large-scale whole genome re-sequencing has now become a reality, especially with the sequencing capacity available in China. An example of this was presented by Xiaowu Wang. They obtained the sequence of *B. rapa* 'Chifu' and have since sequenced a large number of cultivars and found a number of SNPs for glucosinolate metabolism. Quantitative trait loci (QTL) mapping indicates that *MAM3* is likely to be responsible for the high glucosinolate content of some *B. rapa* cultivars. In the future they will be carrying out a similar analysis of *B. oleraceae*.

A further example of massive re-sequencing was delivered by Shuang Yang (Beijing Genomics Institute-Shenzhen (BGI-S), China). He described the way they were using their 128 Illumina instruments to undertake comparative genomics. BGI-S produced the first Asian human genome sequence. He described their deep sequencing of cucumber, silk worms and rice plus their new AQUA software for data analysis. For the last, they have sequenced 25 cultivars and 25 wild types, which basically means EcoTILLING by sequencing! BGI-S is open for collaborations on any topic.

Since this was a workshop, considerable time had been set aside for detailed discussions. This is always a gamble as it can mean long periods of silence unless the audience participates fully! It turned out that there was no fear of this. Excellent discussions took place at the end of the first day and the end of the meeting. The participants kept the questions flowing right through the sessions without even a pause for breath. From the audience responses, it was clear that much was learnt by them, not just about the technologies involved, but also the approaches needed for this particular type of reverse genetics. Gauging the feeling of the meeting, we have put together some notes and an accompanying decision tree to help people decide whether and how to develop TILLING for their own laboratories. We call it 'COAST your way to TILLING'. We hope you find they are useful!

Trevor Wang and **Cristobal Uauy**
John Innes Centre
Norwich, UK

Brad Till
International Atomic Energy Agency/
Food and Agriculture Organisation
Vienna, Austria

Chun-Ming Liu
Institute of Botany
The Chinese Academy of Sciences
Beijing, China

COAST your way to TILLING

C*onsider*

- Is TILLING really right for me?
- Other strategies for functional genomics and reverse genetics. TILLING is just one tool for studying gene function, but it is the best when a range of alleles are needed and good transformation is not available?
- If TILLING is right, check if someone else is already working on your species and can provide a service to you.
- Does your species reproduce sexually? If not consider mutagenizing via tissue culture *in vitro*.
- Is your species a self-fertile inbreeder? If an outcrossing is prevalent, take special measures to prevent it.

O*ptimize*

- The choice of genotype, mutagen, population size and structure can all impact your chances of success.
- Forward screens enrich for deleterious alleles. If you have an interest in a particular biological phenomenon for which there is a good screen, then carry out a forward screen on your plant population using 12-20 M2 plants and use one of these plants for your TILLING population.
- Use good and pure seed to start with. Heterogeneity in seed batches can generate false SNPs.
- Choose genotypes with high seed yields.
- Use literature to find best mutagen or test several mutagens with different modes of action e.g. EMS, azide, γ-rays, not just EMS and MNU.
- Perform a dose-response curve for mutagen. Select a method that works for your species as different species respond differently to different mutagens.
- Keep seed : mutagen ratio constant across different conditions.

A*chieve*

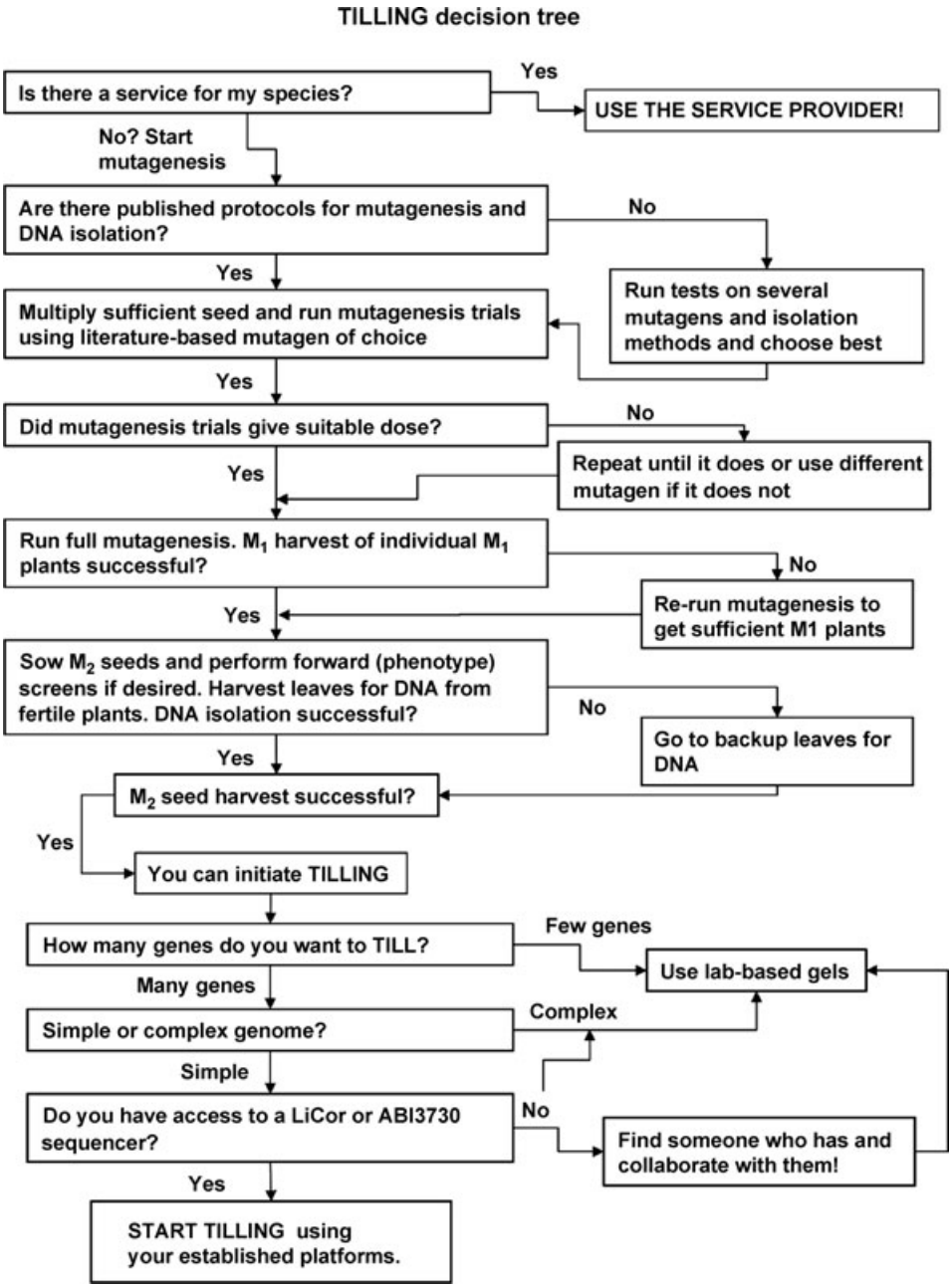
- Mutagenize to get approximately 5 000 M_1 and M_2 plants for your population of a diploid species; polyploids need fewer plants as they tolerate a greater mutation load.
- As guidance use 60–80% germination, but note fertility can drop off very rapidly in some species, so if you can wait, check fertility of trial plants before choosing.
- Keep populations secure (viable). Having put the effort in to produce a population, you do not want to lose it because of incorrect storage. Some species lose viability very quickly when held at room temperature.
- When harvesting material for DNA preparations, save some and store as a backup.
- Try different methods of DNA extraction unless there are tried and tested ones already in the literature. What works for TILLING in one species, may not work in another species.
- The quality and longevity of your genomic DNA preparations, your storage conditions, proper nomenclature for data tracking and your ability to successfully design primers for PCR are all vital aspects as they can make or break a TILLING project.

S*elect*

- There are a variety of choices for platforms for mutation discovery (variables include amplicon size, throughput, cost, accuracy, existing equipment, etc.).
- Technology must fit the need. Do not overcomplicate your process by using inappropriate technology. For example, laboratory run gel systems can be used in many circumstances especially if you only want to examine a few genes, but they are not practical for offering a service.

T*ILLING*

- Do not start TILLING until you have all the pieces in place.
- Scale up and run some test genes to check.
- It is important to continually monitor the quality of your assays and the number and type of mutations you are recovering.


TILLING decision tree
Is there a service for my species?
Yes
USE THE SERVICE PROVIDER!
No? Start mutagenesis
Are there published protocols for mutagenesis and DNA isolation?
No
Run tests on several mutagens and isolation methods and choose best
Yes
Multiply sufficient seed and run mutagenesis trials using literature-based mutagen of choice
Yes
Did mutagenesis trials give suitable dose?
No
Repeat until it does or use different mutagen if it does not
Yes
Run full mutagenesis. M1 harvest of individual M1 plants successful?
No
Re-run mutagenesis to get sufficient M1 plants
Yes
Sow M2 seeds and perform forward (phenotype) screens if desired. Harvest leaves for DNA from fertile plants. DNA isolation successful?
No
Go to backup leaves for DNA
Yes
M2 seed harvest successful?
Yes
You can initiate TILLING
How many genes do you want to TILL?
Few genes
Use lab-based gels
Many genes
Simple or complex genome?
Complex
Simple
Do you have access to a LiCor or ABI3730 sequencer?
No
Find someone who has and collaborate with them!
Yes
START TILLING using your established platforms.