

## **Applications of high-throughput techniques to the understudied crops of Africa**

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### **Summary**

Understudied, underutilized- or orphan- crops provide income for subsistence farmers and staple food for low income consumers in Africa. The major bottlenecks in productivity of these crops are low yield, finger millet (*Eleusine coracana*) and tef (*Eragrostis tef*), poor in nutrition, cassava (*Manihot esculenta*) and enset (*Ensete ventricosum*), and toxicity, cassava and grass pea (*Lathyrus sativus*). Drought, soil acidity and salinity, pests, diseases and weeds contribute to losses in yield. An agricultural revolution is needed to increase food production in Africa. The reverse genetics approach called TILLING (Targeting Induced Local Lesions IN Genomes) can be applied to the understudied crops of Africa. TILLING, a high-throughput and low cost method for the discovery of induced mutations, is currently applied to several crop species. TILLING has been modified as ecoTILLING to identify natural nucleotide polymorphisms. We describe two projects on TILLING and ecoTILLING aimed at finding the limiting factor in tef production.

**Key words:** Under-studied crops, orphan crops, *Eragrostis tef*, TILLING, ecoTILLING

### **Need for Boosting Agricultural Productivity in Africa**

In order to achieve agricultural sustainability the increase in food production should be at least proportional to the rate of population growth. It is, however, expected in near future that with the current level of crop productivity, it might be difficult to feed the population of developing world which is growing at an alarming rate. Crop productivity is mainly affected by a variety of abiotic and biotic stresses. The major abiotic stresses are drought, salinity and acidity. From the total global arable area, a third is affected by salinity and 40% by acidity (Gale, 2002). Biotic factors such as diseases, insects and weeds also reduce crop production tremendously. Crop production could be increased by either expanding the arable area or through intensification, i.e. using improved seed, fertilizer, fungicides, herbicides, irrigation, etc. According to Food and Agriculture Organization, agricultural intensification represents about 80 percent of future increases in crop production in developing countries (FAO, 2002). Based on this goal, crop breeders are focusing towards achieving improved cultivars that produce higher yields and at the same time tolerate to the sub-optimal soil and climatic conditions.

Among plant characters or traits that contributed for higher productivity in the last century, those which alter the architecture of the plant rank first. Architectural changes include alteration in branching pattern and reduction in plant height. The major achievement of Green Revolution in 1960's was due to the introduction of semi-dwarf crop varieties of wheat and rice along with proper crop production packages. These broadly adapted semi-dwarf cultivars were responding

to fertilizer application; which led to tremendous increase in productivity. Currently, a number of genes affecting plant height are identified from major cereal crops including wheat, rice and maize (for review, Wang & Li, 2006). According to the International Food Policy Research Institute, Green Revolution represented the successful adaptation and transfer of scientific revolution in agriculture (IFPRI, 2002). However, since this agricultural revolution did not occur in Africa, crop productivity remains very low.

### Role of Understudied Crops in Africa

Under-studied crops are also known as orphan-, underutilized-, lost-, or disadvantaged crops. These crops play particular role in food security, nutrition, and income generation to resource-poor farmers and consumers in developing countries (Naylor *et al.*, 2004). They also perform better than major crops of the world under extreme soil and climatic conditions prevalent in developing world particularly in Africa. Despite their huge importance, they received little attention by the scientific community. Due to lack of genetic improvement, these crops produce inferior yield in terms of quality and quantity. Moreover, some of these crops produce a variety of toxins which are hazardous to humans if consumed before special processing procedure. The understudied crops are mainly grouped under cereals (e.g. millet, tef), legumes (bambara groundnut, grass pea), and root crops (cassava, yam, enset). Brief descriptions are given below for some of them:

Finger millet (*Eleusine coracana* L.) is grown on a total area of about 4 million hectares in more than 25 countries in Africa and Asia. The plant is tolerant to drought. The seed of finger millet contains valuable amino acid called methionine (NRC, 1996), which is lacking in the diets of hundreds of millions of the poor who live on starchy staples such as cassava. Finger millet is also a popular food among diabetic patients because of its slow digestion.

Tef [*Eragrostis tef* (Zucc.) Trotter] is grown annually on over 2.5 million hectares of land mainly in Ethiopia. The plant is tolerant to abiotic stresses especially to poorly drained soils where other crops such as maize and wheat could not withstand. In addition, the seeds of tef produce healthy food because they do not contain gluten for which large portion of the population are allergic (Spaenij-Dekking *et al.*, 2005). Unlike other cereals, the seeds of tef can be stored easily without losing viability under local storage conditions, since it is not attacked by storage pests (Ketema, 1997).

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is an annual legume crop grown for human consumption. The seeds of bambara groundnut are known as a complete food because they contain sufficient quantities of protein, carbohydrate and fat. The average composition of the seed is 63 percent carbohydrate, 19 percent protein, and 6.5 percent oil (NRC, 2006).

Grass pea (*Lathyrus sativus*) is another legume plant commonly grown for human consumption in Asia and Africa. The plant is extremely tolerant to drought and is considered as an insurance crop since it produces reliable yields when all other crops fail. Like other grain legumes grass pea is a source of protein particularly for resource poor farmers and consumers. However, the seeds of grass pea contain a neuron-toxic substance called ODAP [ $\beta$ -N-Oxalyl-L- $\alpha$ ,  $\beta$ -diaminopropanoic acid (Yan *et al.*, 2006)]. ODAP is the cause of the disease known as neurolathyrism, a neurodegenerative disease that causes paralysis of the lower body. Serious neurolathyrism epidemics have been reported during famines when grass pea is the only food source (Getahun *et al.*, 2003).

Cassava [manioc; *Manihot esculenta* Crantz] is staple food for about a billion people (<http://www.fao.org/newsroom/en/news/2008/1000899/index.html>, accessed 31 July 2009). The plant is tolerant to drought and also performs better than other crops on soils with poor nutrients. The major problems related to cassava are their very low protein content and the roots contain poisonous compounds called cyanogenic glycosides (CG) which liberate cyanide (Ceballos *et al.*, 2004). Konzo is a paralytic disease associated with consumption of insufficiently processed cassava.

Yam (*Dioscorea* sp.) represents different species under genus *Dioscorea*. It is grown on about 5

million hectares of land world wide (IITA, <http://www.iita.org/>) and staple food in west Africa. The roots are the edible part and looks like sweet potato (*Ipomoea batatas*) although they are not taxonomically related.

Enset (*Ensete ventricosum*) is commonly known as 'false banana' for its close resemblance to the domesticated banana plant. Unlike banana where the fruit is consumed, in enset the pseudostem and the underground corm are the edible parts. Enset is the major food for over 10 million people in densely populated regions of Ethiopia. The plant is considered as an extremely drought tolerant and adapts to different soil types (Brandt *et al.*, 1997). Since enset flour is rich in starch but not in other essential nutrients enset-based diets need heavy supplementation.

In general the major bottlenecks affecting the productivity of under-researched crops are genetic traits such as low yield (tef, millet), poor in nutrition (cassava, enset), and production of toxic substances (cassava, grass pea). Environmental factors such as drought, soil acidity and salinity, pests, diseases and weeds also contribute for large loss in quality and quantity of the yield. Hence, agricultural revolution is needed to boost the productivity these crops in order to feed the ever increasing population in Africa.

### **TILLING: a High-throughput Technique of Crop Improvement**

Modern crop breeding utilizes different techniques that are mostly dependent on molecular markers. Genetic engineering is also widely exploited to transfer a single or multiple gene(s) of interest within or across species. As a result, plants which are tolerant to a multitude of environmental stresses or those with improved nutritional qualities are obtained (Bhatnagar-Mathur *et al.*, 2008). However, transgenic crops currently face many protests particularly in Africa where research and field testing are strictly forbidden.

This calls for an alternative non-transgenic approach of crop improvement in order to solve crisis in food production. A recently discovered robust technique called TILLING (Targeting Induced Local Lesions IN Genomes) is an attractive option to consider. TILLING is a reverse genetic technique that uses traditional mutagenesis method to create population that are subjected to high throughput screens for the discovery of mutations. The technique is proved to be efficiently working in diverse crops with different genome size and ploidy level including maize (Till *et al.*, 2004), barley (Caldwell *et al.*, 2004), wheat (Slade *et al.*, 2005), rice (Till *et al.*, 2007), soybean (Cooper *et al.*, 2008), pea (Triques *et al.*, 2007), and sorghum (Xin *et al.*, 2008).

TILLING has the following advantages over other improvement techniques, i) it produces a spectrum of allelic mutations that are useful for genetic analysis, ii) it is applicable to any organism, iii) mutations difficult to know by forward genetics could be revealed by TILLING since it can focus at a particular gene of interest, and iv) it is a non-transgenic method, hence the product is readily accepted by all members of the society. In general, TILLING detects rapidly point mutations in the population independence of genome size, reproductive system or generation time.

#### **The TILLING method**

Step-by-step procedures of TILLING are shown below (also depicted in Fig. 1):

- Identify proper mutagen: EMS (Ethyl methanesulfonate) is widely used as a mutagen for many crops but sodium azide is the preferred mutagen for barely (Caldwell *et al.*, 2004).
- Select tissue for mutagenesis: Seeds are mostly used for mutagenesis except in maize where pollen grains are directly exposed to the mutagen (Till *et al.*, 2004).
- Generate population: The size of mutagenized population depends on genome size, objective of the study, and availability of resources. Samples for DNA isolation are



mostly collected from  $M_2$  population.

- DNA extraction: Genomic DNA is isolated from  $M_2$  samples. Once the concentration of DNA is determined, pools of four or eight are made.
- Identify and isolate gene of interest: Full-length genomic sequence is made available for the selected gene(s).
- Primers and PCR amplification: Labelled primers are designed using two IRDyes (infrared dyes). PCR amplification is followed by a denaturation and slow-annealing steps in order to form a hetero-duplex at the position of mutation or polymorphism.
- Enzymatic assay: Many investigators utilize an enzyme isolated from a celery known as CEL I (Caldwell *et al.*, 2004; Cooper *et al.*, 2008; Slade *et al.*, 2005; Till *et al.*, 2004, 2007; Xin *et al.*, 2008), while others use Endo I (Triques *et al.*, 2007) or an extract from brassica petiole (Sato *et al.*, 2006). More recently, the Next Generation Sequencing particularly the 454 FLX system is also efficiently utilized in mutation detection (Rigola *et al.*, 2009).
- Mutation discovery: Mutation detection is made by running the samples on the LiCOR after enzymatic assay. Once the mutation is discovered in pools, steps from PCR amplification to mutation discovery are repeated using DNA isolated from individual line.

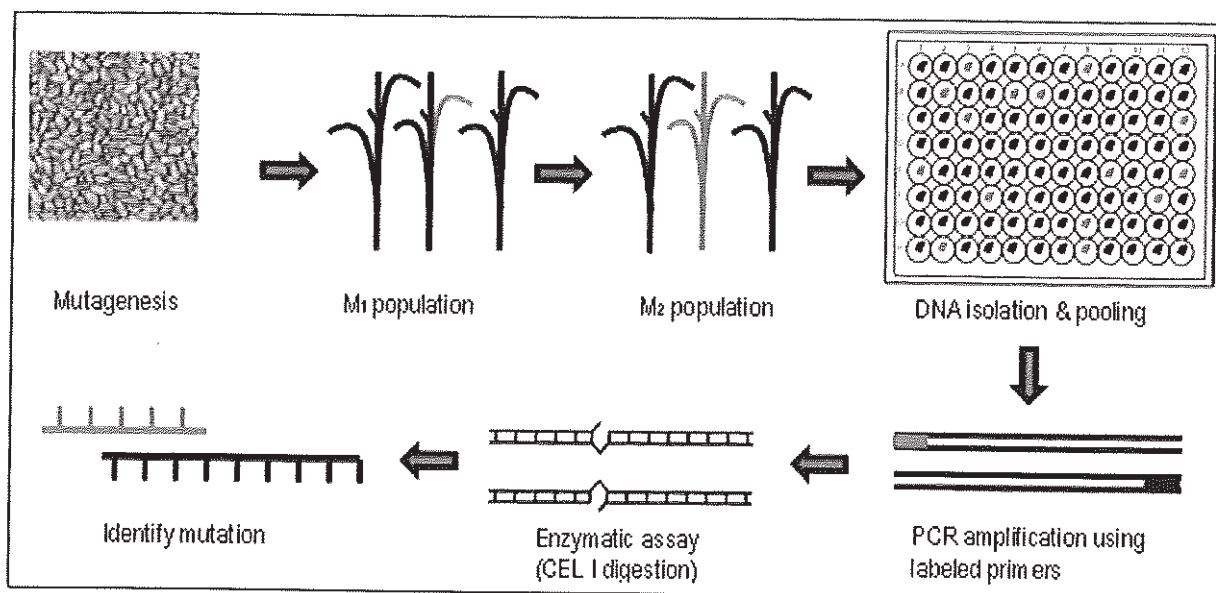


Fig.1. The procedure of TILLING.

### EcoTILLING in the Detection of Natural Variations

EcoTILLING refers to modified form of TILLING where polymorphisms in natural population are detected (Comai *et al.*, 2004). Like TILLING, ecoTILLING is also effectively applied in many crops including mung bean (Barkley *et al.*, 2008), sugarcane (McIntyre *et al.*, 2006), and peanut (Ramos *et al.*, 2009).

### Application of TILLING and ecoTILLING to the understudied crops

The TILLING and ecoTILLING projects on cassava and banana are currently being executed in the collaboration between the Joint FAO/IAEA Programme and the International Institute of Tropical Agriculture (Bradley Till and Melaku Gedil, personal communication). Below we present

the status and perspectives of the Tef TILLING and ecoTILLING projects.

### The Tef TILLING and EcoTILLING Projects

Although tef is critical in the economy of the least developed country like Ethiopia, its productivity is too low compared to other cereal crops. The major bottleneck to low productivity is its susceptibility to lodging particularly when occurs during grain filling period. Lodging refers to the state of permanent displacement of the stem or stalk from its upright position. Tef has a tall and tender stem that is susceptible to damage by wind and rain. Furthermore, lodging poses problems for the modernization of production as it constrains such factors as irrigation and mechanical harvesting. As a consequence the yield from the crop is severely reduced in terms of total grain yield and quality.

Research made on tef so far focused on the conventional breeding techniques such as selection and hybridization. As a result, a number of tef cultivars were released to the farming community. However, no single lodging tolerant cultivar is yet obtained. On the other hand, the semi-dwarf wheat and rice cultivars that were developed during the Green Revolution in 1960's and 1970's and then after are semi-dwarf in stature and are tolerant to lodging. Experiences from wheat and rice indicate that semi-dwarf cultivars have high potential in boosting crop yields. The prominent semi-dwarf cultivars harbour mutation in the gene *Reduced height-1* (Rht-B1 and Rht-D1) for wheat (Peng *et al.*, 1999), and *semi-dwarf (sd-1)* for rice (Spielmeyer *et al.*, 2002). Rht-B1 and Rht-D1 are the orthologs of Gibberellin Insensitive (GAI) gene while Sd-1 belongs to Gibberellin 20-oxidase (Peng *et al.*, 1999, Spielmeyer *et al.*, 2002).

The Tef TILLING project, based at the University of Bern in Switzerland, was initiated with financial supports from Syngenta Foundation for Sustainable Agriculture and University of Bern. The main goal of the project is to obtain semi-dwarf tef lines that are resistant to lodging. The project has so far generated over 10000 M<sub>1</sub> and 6000 M<sub>2</sub> tef populations mutagenized with EMS, a chemical known to induce point mutations. Leaf samples were collected from 2–3 weeks old M<sub>2</sub> seedlings and the seeds of M<sub>3</sub> lines were harvested. DNA isolation was made for all M<sub>2</sub> samples. Two genes known to influence plant height in rice, namely HTD1 (HIGH-TILLERING DWARF1, Zou *et al.*, 2006) and DWARF4 (Sakamoto *et al.*, 2006) were isolated from tef. Based on the sequence information each of these genes is represented by two copies having high homology in the exon region but divergent in the intron region. The divergent region is used to design copy specific primers in order to amplify only one copy at a time. IRDye 700 and IRDye 800 are used to label the forward and reverse primers, respectively. The PCR products are first tested on the gel electrophoresis and later on the LiCOR gel after enzymatic assay. Currently we are at the last stage of optimizing the amount and concentration of DNA to be used, the size of pools, etc before embarking large-scale screening of the population.

Since substantial amount of genetic differences were reported among tef accessions (Assefa *et al.*, 2001), we are also interested to utilize ecoTILLING in order to investigate natural polymorphisms. Over 500 accessions collected from different tef growing regions were grown in the Green House in Bern. Genomic DNA from leaf samples were isolated for all samples and at the present time the samples are ready for high-throughput screening using the two genes indicated above.

The major problems related to TILLING and ecoTILLING in tef is related to the polyploidy nature of the plant. Tef is an allotetraploid with  $2n=4x=40$ . Since each gene is present at least in two copies, PCR amplification and detection of a single copy is difficult. In order to tackle this problem, we first identify how many copies of a gene are present in the genome. Then, we design primer(s) that can preferentially amplify one copy at a time.

In parallel, in order to identify semi-dwarf tef plants 10 lines each from 4000 M<sub>2</sub> population were grown for about four weeks in the greenhouses and growth rooms at Syngenta in Stein and Institute of Plant Sciences in Bern where many environmental factors are controlled. Using visual

observation several candidate mutants were selected. At the moment, they are closely investigated for fertility, inheritance of the traits, etc. The picture of one candidate is shown in Fig. 2.



Fig. 2. The semi-dwarf tef line (right) as compared to the original line before mutagenesis. Several candidate semi-dwarf lines were obtained from visually screening over 4000 mutagenized population under environmentally controlled conditions.

### Conclusions

Understudied crops provide food for resource poor farmers and consumers in Africa. They also grow under extreme environmental conditions, many of them poorly suited to major crops of the world. Since Green Revolution did not occur in Africa, the continent did not benefit from the positive effects of this agricultural revolution that boosted the productivity of crops in other parts of the world. The next Green Revolution for Africa needs to include these locally adapted crops that are mostly known as orphan- or understudied-crops. The implementation of TILLING and ecoTILLING to the understudied crops has many advantages. The techniques do not require prior knowledge of the genome sequence except for the gene of interest. The other advantage is for the polyploidy species like finger millet and tef where most mutations do not show obvious phenotypes



due to the existence of un-mutated gene copies. Since TILLING and ecoTILLING reveal many allelic mutations, through double or multiple crossings expected mutant phenotypes could be obtained.

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