

# Developments in Mutation Assisted Plant Breeding

## A. Introduction

Spontaneous mutation, the naturally occurring heritable change to genetic material which played a pivotal role in biological evolution and formed the basis for speciation and domestication of both crops and animals, can be artificially induced and supports the maintenance of biodiversity. Since the discovery of X-ray and other forms of radiation at the end of the 19th century and the ensuing demonstration of the ability of these forms of nuclear energy to alter genetic material, scientists have routinely used different types of ionizing radiation to create variants of crops [I-1].

There have been more than 2700 officially released mutant varieties from 170 different plant species in more than 60 countries throughout the world (Fig. I-1) that not only increase biodiversity, but also provide breeding material for conventional plant breeding, thus directly contributing to the conservation and use of plant genetic resources. Hundreds of millions of hectares of higher yielding or more disease-resistant crops have been developed annually through induced mutations and released to smallholders. These mutant varieties enhance rural income, improve human nutrition and contribute to environmentally sustainable food security in the world. Close to 90% of these officially released mutant varieties were produced using radiation (Fig. I-2) and contribute billions of dollars of additional income to farmers annually [I-2].<sup>1</sup>

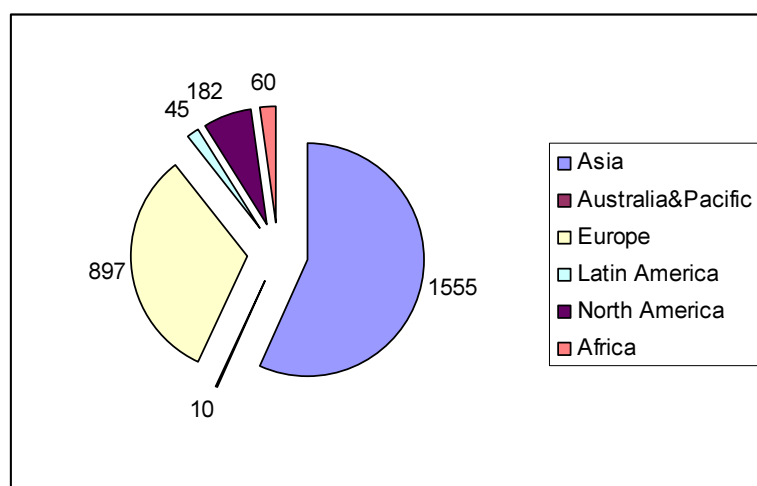
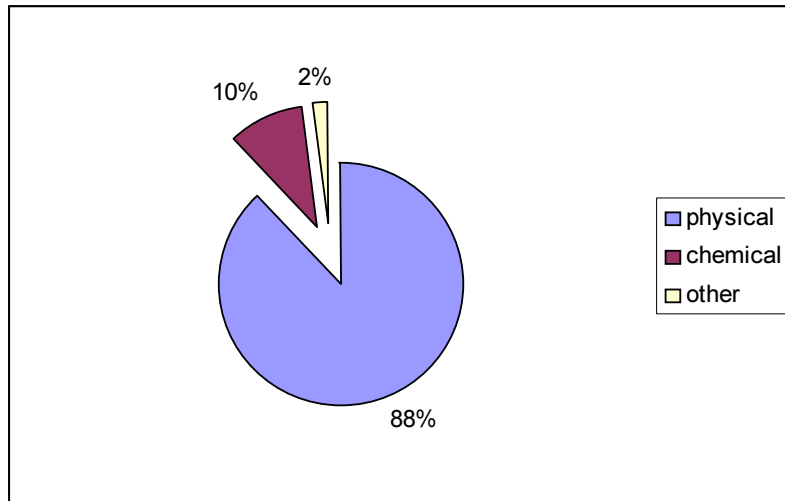


FIG. I-1. Number of mutant varieties developed in different regions of the world (FAO/IAEA Mutant Varieties and Genetic Stock Database <http://mvgs.iaea.org/>).

<sup>1</sup> The FAO/IAEA Joint Division is working in partnership with national agricultural authorities, farmers, and global research institutes such as the International Rice Research Institute (IRRI), with headquarters in the Philippines, to introduce new mutant varieties of wheat, rice and cassava species, in order to increase agricultural efficiency, particularly in marginal lands featuring drought and poor soil fertility.



*FIG. 1-2. Comparative usage of different types of mutagens. The majority of mutant varieties are developed through irradiation (e.g. fast neutrons, X-rays, more than 64% through gamma irradiation).*

Since the early 1980s, there has been a surge in the applications of recombinant DNA technologies in the quest for answers to several biological questions in health, agriculture and industry. This has brought about an unprecedented escalation in the volume of information on the ‘building blocks’ of heredity (i.e. DNA sequence information), which is now available in the public domain. The DNA sequences of many organisms including the human organism (e.g. the human genome project) and several crop species have been published. This is referred to as the genomics era, a term coined to reflect the availability of information spanning the total genetic make-up of an organism, the genome. The next goal in genomic studies is to unlock information contained in the genomes of humans, animals, and plants, identifying and ascribing functions to those parts of the genetic make-up; this will have far reaching scientific implications and commercial potential. At the same time, various molecular and genomic tools and techniques have been developed and have substantially transformed the landscape of biological research. Efforts towards crop improvement have also entered this new era, increasingly using both genomic knowledge and various molecular tools. Coinciding with these developments, an expansion in the research and application of induced mutations for crop improvement has also taken place in scientific communities throughout the world.

Mutation induction continues to contribute to crop improvement, using physical mutagens such as gamma ray, X-ray, fast neutron, and chemical mutagens such as EMS (ethyl-methane-sulphonate) and sodium azides. Recently, new physical mutagens, such as ion beam radiation and cosmic rays, have been proven to be effective for inducing mutations.

Below is an overview of recent developments related to plant mutation breeding; induced mutations for harnessing genomics resources; bio-/molecular technologies for enhancing the efficiency of mutation induction and utilization; and ion beam radiation as a novel mutation induction technique.

## **B. Mutant Stocks as Gene Repositories**

Generations of induced mutants have long been driven by plant breeders, who would then use them directly or indirectly (through further cross breeding) in the process of developing new varieties. The plant breeder’s ability to improve a crop is as good as the available genetic variation, which can be exploited in cross breeding and other forms of selection. Mutation induction offers the possibility to

induce such variations where the desired genetic variation is lacking. Since the genomic region critical for a trait, known as the gene, can be identified by the deductive process of inducing a series of mutants, identifying which traits have been modified and relating such observed modifications to changes in genomic regions of such mutants compared to the “normal” types is important. The mutated genomic region that exerts control over the trait in question is therefore identified as the gene responsible for the expression of the trait. The mutant genetic resources as one of the outputs of mutation induction is an indispensable tool not only in plant breeding research but also in fundamental genetic research activities related to gene discovery and gene function analyses.

Significant efforts are currently being made not only in the generation of genetic variations through induced mutations but also in the systematic characterization of the induced mutants for observed phenotypic and genomic changes and the cataloguing of the variations in searchable databases. Many of these databases are available to interested parties.

Climate change may well lead to significant losses of genetic diversity within species critical for food and agriculture. Roughly 20 - 30% of species assessed are likely to be at high risk of extinction, if global mean temperature exceeds 2 - 3°C above pre-industrial levels. These include crops' wild relatives, which are essential to increasing agricultural productivity, but also serve as the foundation for adaptation strategies required for adjusting to abiotic changes such as heat, drought and salinity as well as pests and diseases. Loss of genetic diversity would have important negative consequences locally as well as internationally, because key traits for climate change and variability adaptation may be lost forever. Mutation induction can help to meet this challenge.

Most national governments have also established germplasm collections that contain sizable amounts of characterized mutant stocks that are distributed to plant breeders and geneticists. The U.S. Department of Agriculture, Agricultural Research Service (USDA/ARS) runs several germplasm repositories (GRIN) that also include mutant genetic stocks under the National Plant Germplasm System (<http://www.ars-grin.gov/npgs/holdings.html>) and is considered to be one of the most comprehensive and best organized collections.<sup>2</sup>

In addition to these genetic resources with mostly single point mutations, another type of mutant genetic resource, radiation hybrids (RH), has been developed for genomic research. RHs are produced by exposing somatic cells to lethal doses of gamma radiation or X-ray, in order to fragment the chromosomes. They are then rescued by introduction into host cells, which are subsequently fused with suitable recipient cells for the assessment of their expressions providing unique materials for the establishment of physical maps, a process known as radiation hybrid mapping. RH maps have been developed in a number of crops, such as barley, maize, wheat and cotton for gene discovery and detailed linkage analysis. This may soon lead to the identification and transfer of genes affecting useful agronomic, quality and stress tolerance traits.

## **C. Nuclear Methodologies: Enhancing Efficiency through the Application of Bio-/Molecular Technologies**

### **C.1. Novel molecular biology strategies for enhancing the efficiency of mutation induction techniques**

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<sup>2</sup> Information in the FAO/IAEA Database of Mutant Variety and Genetic Stock has been voluntarily contributed by national agricultural research institutes and breeders such as the Radiation Breeding Institute of Japan and the Zhejiang University in China (an IAEA Collaborating Centre). For the IAEA mutant germplasm repository (MGR), please see <http://mvgs.iaea.org>.

A major bottleneck in the routine application of induced mutagenesis to both crop improvement and genomic research (through forward and reverse genetics) remains the drudgery of producing, handling and assaying the requisite large mutant populations. This is because mutant events usually occur in low frequencies and detection therefore requires the creation of large mutant populations. Molecular biology strategies, by permitting the querying of the genome, provide neutral tools that are independent of environmental or other extraneous factors for characterizing living organisms. One molecular biology strategy, reverse genetics—the use of modifications at the molecular level to predict phenotypes, holds great promise for reducing the number of putative mutants for expensive field trials or laboratory analysis, since plants without any alteration in the target gene could be effectively excluded from those tests.

Recent advances in genomics, especially publicly available genomics resources, have permitted the use of a high throughput platform such as “Targeted Induced Local Lesions in Genomes (TILLING)” in the rapid evaluation of mutant stocks for specific genomic sequence alterations. TILLING is currently being used by many research groups in the identification of mutation events. In practice, the knowledge of the frequency of induced mutations in a population allows the calculation of the optimal population size, avoiding larger populations than necessary to be screened. Therefore, a pre-test of mutation frequency is helpful before starting whole population screening. Populations with too low a mutation frequency should be discarded.

In recognition of the utility of this method in high throughput mutation discovery, many laboratories have set up the TILLING platform and a number of laboratories also provide TILLING services for specific plants.

Another problem is the need to have the mutated segment in a homozygous state so that the mutation, usually recessive, could manifest as a phenotype. Another major difficulty is the inherent problem of chimeras, a problem that is exacerbated in vegetatively propagated plants. A number of *in vitro* techniques have been shown to circumvent or significantly mitigate these bottlenecks to induced mutations. These include cell suspension cultures including somatic embryogenesis; doubled haploid production and rapid *in vitro* multiplication.

### **C.1.1. Cell and tissue culture**

Cellular and tissue biology strategies enhance efficiency of induced mutation in crops. Related to the problem of production and subsequent detection of mutation events in homozygous genomes are the confounding effects of chimeras, especially in vegetatively propagated crops. This problem is mitigated through several cycles of vegetative regeneration (both *in vitro* and *in vivo*) of the mutagenised vegetative propagule (i.e. plant material used for the purpose of plant propagation, e.g. any highly meristematic part such as root and stem ends or buds, but also cuttings, leaf sections, or any number of other plant parts that could be induced to regenerate whole plants *in vitro*).

This is also expensive in terms of time and resources and could be circumvented through the use of cell suspension cultures as starting materials for inducing mutations. Cell suspension cultures take advantage of the potential of each plant cell to regenerate into a whole plant, a phenomenon known as totipotency. In practice, this involves the production of cell lines from callus followed by the regeneration of plantlets through somatic embryogenesis. Typically, single cells and small cell aggregates are cultured; these proliferate and complete a growth cycle while suspended in a liquid medium. Since this technique was demonstrated in 1956 with *Phaseolus vulgaris*, reproducible protocols have been validated for other plant species [I-10]. This ability to culture individual plant cells, from which whole plantlets will arise, permits the treatment of individual cells with mutagens. The resulting plantlets are genetically similar leading to significant gains in time.

Where protocols for somatic embryogenesis, through cell lines or friable embryogenic calli, for instance, are not available, plantlets could also be regenerated but at relatively lowered levels of homozygosity and enhanced levels of chimeral sectors through in vitro nodal segments. While this is not optimal, it is still better than using stem cuttings or other tissues for crops that due to biological or genetic characteristics can only be vegetatively propagated and for which microspore cultures followed by chromosome doubling are impracticable. If this route is taken, due consideration must be given to planning strategies for efficient dissociation of chimeras. Where multi-cellular meristematic tissues have been used as starting materials for the induction of mutations, several cycles of regenerations, with M1V4 being the minimum, are required to dissociate chimeras in order to approximate the homohistont state.

### **C.1.2. Doubled haploid production**

Another bottleneck to induced crop mutations relates to quality and the inherent recessive nature of mutations. This leads to the masking of the mutation events in the appearance of the mutants by the dominant allele at the same gene locus. In a heterozygous background therefore, phenotypic manifestations of mutations are practically impossible to detect in the early progenies necessitating several cycles of crossing the plant with itself in order to produce homozygous recessives that express the recessive phenotype.

Again, totipotency is exploited in the regeneration of doubled haploids (DHs), when the chromosome number of gametic cells, i.e. pollens or anthers and egg cells, is doubled prior to regeneration of a plant [I-6] to mitigate this problem. This process could be incorporated into induced mutagenesis by the treatment of these gametic cells prior to regeneration of the doubled haploids. With spontaneous and/or induced doubling of the haploid chromosomes, homozygous individuals are produced, availing the researcher of the most rapid route to attaining homozygosity without having to cross the plant with itself [I-13]. By facilitating the possibility of targeting either the haploid or doubled haploid cells for mutation treatment, a mutation is captured in a homozygous, pure line [I-13]. These mutants are homozygous for all loci including the mutated segments of the genome being targeted for modification and subsequent detection. For seed propagated crops, doubled haploid strategies provide the fastest method for achieving homozygosity, as compared to self-pollination. The savings in time and cost are significant as recessive mutations usually are not detectable till the first self-pollination generation or later generations. Rapid advances in cellular and tissue biology techniques have resulted in the availability of reproducible DH protocols for over 250 plant species [I-9] covering most plant genera. The Agency has, through its coordinated research activities, supported the development of easily applicable DH protocols for many crop species. The DH methodology has been successfully used to expedite the pace for generating true breeding mutants in crops such as barley, wheat, rice. Salt tolerant wheat was produced in China by combining mutagenesis with anther culture and at the Agency's laboratories, DH was also used to generate a semi-dwarf (and hence lodging resistant) rice mutant from a salt tolerant but uncultivated wild relative of rice [I-3].

### **C.1.3. Ion Beam Mutation**

Ion beams have been widely used in the research on material surface modification since the 1970s. Their application for mutation induction was started with low-energy ions in China in the late 1980s and with heavy ions in Japan in the early 1990s. While ion beam technology has been used for food crop improvement in China, it has been more extensively used for floriculture plants in Japan.

Ion beams as a mutagen are different from other physical mutagens such as gamma or X-rays in that they not only involve energy transfer (as gamma or X-rays), but also mass deposition and charge exchange; hence could result in complex DNA damage and changes that are not found when gamma or X-rays are used (high percentage of double strand breaks and subsequent chromosome aberrations). Ion beams are produced by particle accelerators, i.e. cyclotrons. Figure I-3 is a schematic view of the E5B beam line available in the RIKEN Accelerator Research Facility (RARF) Japan.

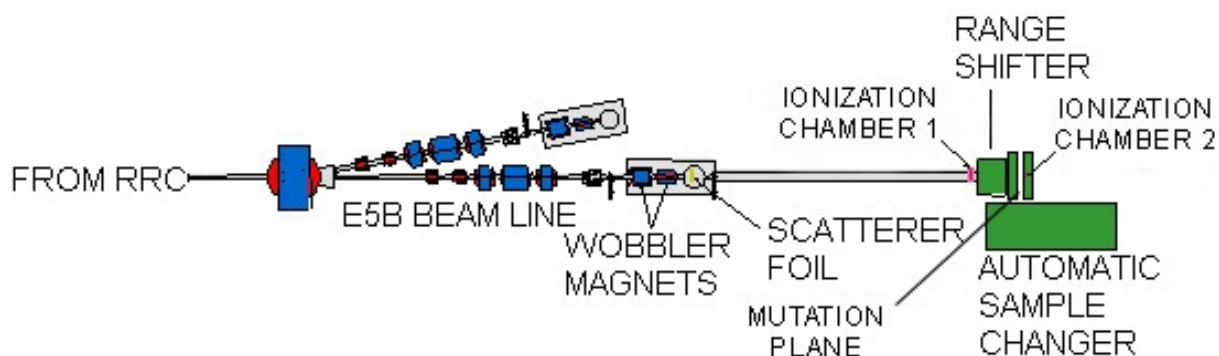


FIG. I-3. The schematic view of E5B beam line (RRC = RIKEN Ring Cyclotron) See Table I-1 for examples of heavy ions used in biological research [I-17].

Typical ions used for irradiation on biological samples are neon-20, nitrogen-14, carbon-12, lithium-7, argon-40, iron-56 (Table I-1). They have different energy levels and linear energy transfer (LET), ionization densities which correlate to the complexity of DNA damage, and different ranges of penetration (Fig. I-4). It is possible to modulate the treatment of plant material with one species of ion at different LETs by passing the ions through a combination of absorbers – since changes in the LET of ion species occur as they pass through matter [I-17].

| Ion              | Energy |      | Charge | Range in Water (mm) | LET (keV/ $\mu$ m) |
|------------------|--------|------|--------|---------------------|--------------------|
|                  | MeV/u  | GeV  |        |                     |                    |
| $^{12}\text{C}$  | 135    | 1.62 | 6+     | 43                  | 22.5               |
| $^{14}\text{N}$  | 135    | 1.89 | 7+     | 34                  | 26.3               |
| $^{20}\text{Ne}$ | 135    | 2.70 | 10+    | 23                  | 61.1               |
| $^{40}\text{Ar}$ | 95     | 3.80 | 17+    | 8                   | 280.0              |
| $^{56}\text{Fe}$ | 90     | 5.04 | 24+    | 4                   | 624.0              |

Table I-1. Heavy ions for biological research in RIKEN Accelerator Research Facility (RARF) [I-17].

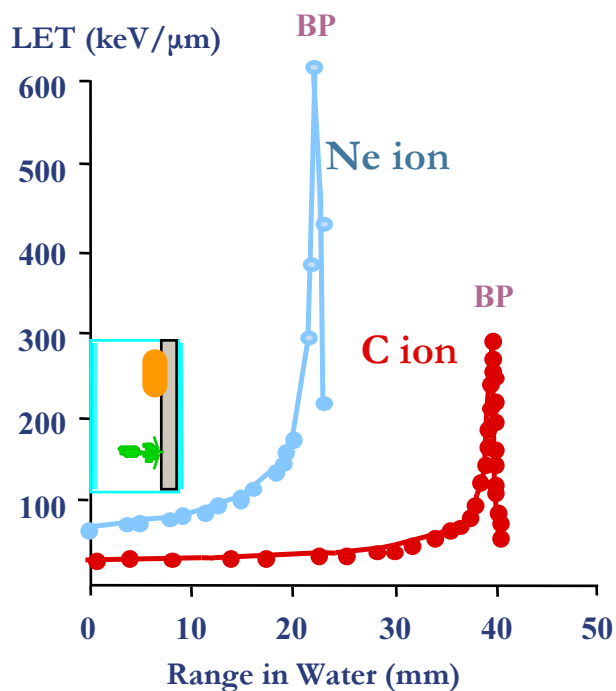


FIG. I-4. After a beam with sufficient energy penetrates a plantlet and/or plant tissue with rather low and uniform LET, the LET will then drastically increase towards the end of the track which is known as the Bragg peak (BP) [I-17].

Studies have shown that the biological effect of ion beam radiation is dependant on absorption doses and LET values but independent of ion species [I-17], which means that the treatment of carbon-12 would produce similar biological effect on rice seeds as neon-20 if the same dose (say 50 Gy) and same LET (say 30 keV/μm) is applied. DNA double strand breaks are believed to be the most important consequence of ion beam radiation. Very complex repair mechanisms have been unveiled but are prone to errors due to double-strand breaks and lead to deletions, insertions, inversions and translocations. Studies on the mutant gene alleles induced by ion beam radiation showed that most mutations are deletions and that the size of DNA deletion is LET-dependant.

Most complex DNA damage caused by the intricate set of effects of heavy ion beams (HIB) escapes the repair efforts and thus is described as more biologically effective and mutagenic than X-rays and gamma rays. A wider mutation spectrum and less collateral physiological damage (i.e. effect on plant survival and growth) is commonly reported for ion beam radiation as compared to other mutagens, which is considered an important advantage.

In China, 23 new rice and wheat mutant varieties have been bred using ion beam technology and released for large scale commercial production (more than one million ha per annum). The wheat variety 'Wanmai 54' displayed excellent resistance to head scab disease and rust disease and recorded the highest yield in the national new wheat variety yield trial (2003-2007), with yield increases over control variety of 7~10.6%. In Japan, ion beam technology has been used for generating mutants for a vast number of plant species by various researchers; for example, a consortium of more than 90 user groups was established to utilize the ion beam technology available in RARF (Japan). Six new flower varieties have been developed using this technology and marketed in Canada, Japan (Fig. I-5), USA, and the EU since 2002.



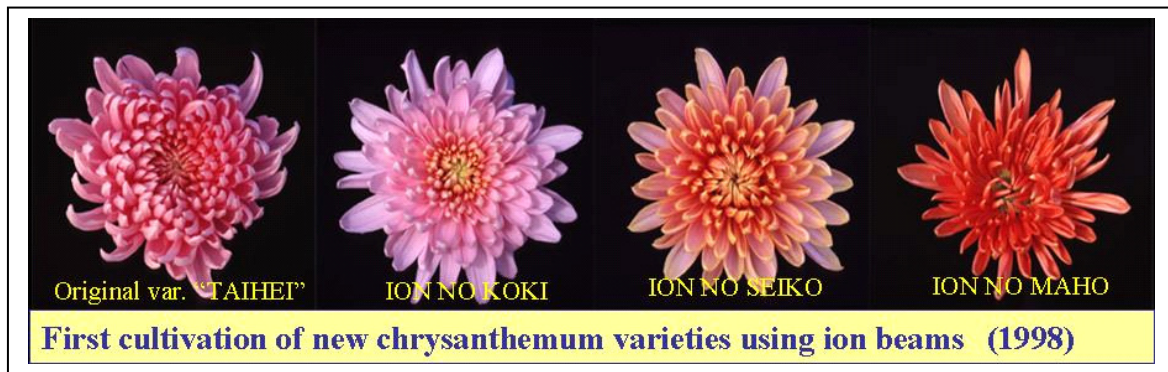


FIG. I-5: First cultivation in 1998 of new chrysanthemum varieties using ion beams (courtesy of Dr. A. Takana, ISIMP2008/342).

## D. Future Perspectives

With the imminent threats posed by global climate change to crop production and the ever increasing and more sophisticated demands of agricultural products, crop improvement efforts have to be more powerful and precise in developing new crop varieties. Breeders therefore require tools that permit achieving subtle changes to the genetic make-up of otherwise superior crop varieties e.g. high yielding but lacking in specific quality traits, and yet leaving the genome largely intact in order not to disturb already stacked alleles of genes. The availability of genomics information in the public domain coupled with recent advances in molecular and cellular biology techniques have paved the way for transforming old mutation techniques into state of the art technology for both crop improvement and basic genomics research.

Development of novel and more efficient genomic tools have become routine and the pursuit of new physical mutagens continues. Some technologies are already in place and when integrated into mutation research, they will greatly increase the efficiency and application of mutation techniques in plant research. For example, the next generation sequencing technologies, e.g. Roche 454 Genome Sequencer-FLX™ and Applied Biosystems SOLiD™ instruments, have the potential to reduce the cost of genome sequencing by several magnitudes, and simplify the process of mutation detection, the key point in mutation research and application programmes. In particular, they will enable the identification of mutant genes underlying important quantitative traits such as drought tolerance and yield, something that is still very difficult, if not impossible with traditional means.

Exposure of organisms to outer space conditions such as microgravity, vacuum, ultra-clean environment, cosmic rays, have allowed some countries, since the 1960s, to pursue programmes of “space breeding”. Whilst it is impractical to run commercial “space breeding” programmes, experiments in a simulated space environment are being conducted on earth using accelerator generated mixed high-energy particles that mimic secondary cosmic rays, including pion, meson, muon, positron, electron, photon and proton radiations. These may lead to the discovery of new physical mutagens that are more effective or have unique properties.



## E. Conclusion

In conclusion, by facilitating the direct querying of target genes for changes, molecular biology techniques will significantly obviate the need for field trialling large populations. Additionally, robust; cheap and easy to use analytical methods will be ‘hooked’ up to these novel methods to enhance efficiency of the delivery processes. Cellular biology techniques will address the bottlenecks imposed by the need to rapidly generate large mutant populations of suitable genetic backgrounds (homozygous for the mutation events, and devoid of chimeras). New, space-age technologies are being developed for mutation induction. The Agency is working with a network of experts with the objective of using a set of globally important food crops to validate identified relevant novel techniques and build these into modular pipelines to serve as technology packages for induced crop mutations. Thus, mutation assisted plant breeding will play a crucial role in the generation of ‘designer crop varieties’ to address the uncertainties of global climate variability and change, and the challenges of global food insecurity.

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