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The Role of Induced Plant Mutations in the Present Era

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ABSTRACT

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. 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. 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.

Keywords: agricultural biostimulants, compost-stabilized waste, humification indices, organic carbon fractions, soil microbial populations

Abbreviations: **AHAS**, altered acetohydoxyacid synthase; **DH**, double haploids; **EMS**, ethyl methanesulphonate; **HMWG**, high molecular weight glutenins; **TILLING**, targeted induced local lesions in genomes

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INTRODUCTION

The application of mutation techniques by using different agents of physical and chemical nature has generated a vast amount of genetic variability and has played a significant role in plant breeding and genetic studies. The widespread use of induced mutants in plant breeding programmes throughout the world has led to the official release of more than 3000 plant mutant varieties from 170 different plant species in more than 60 countries throughout the world (IAEA 2011); the developed varieties increase biodiversity and provide breeding material for conventional plant breeding, thus directly contributing to the conservation and use of plant genetic resources.

A large number of these varieties (including cereals, pulses, oil, root and tuber crops, and ornamentals) have been released in developing countries, resulting in enormous positive economic impacts.

During the last decades, with the unfolding of new biological fields such as genomics and functional genomics, bioinformatics and the development of new technologies based on these sciences, there has been an increased interest in induced mutations within the scientific community.

Induced mutations are now widely used for the discovery of genes controlling important traits and understanding the functions and mechanisms of actions of these genes, in addition to develop improved crop varieties. Progress is also being made in deciphering the biological nature of DNA damage and repair.

THE ROLE OF INDUCED MUTATIONS IN WORLD FOOD SECURITY

Hundreds of millions of hectares have been cultivated annually with varieties improved by 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

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90% of these officially released mutant varieties were produced using radiation and contribute billions of dollars of additional income to farmers annually (Ahloowalia *et al.* 2005).

Food security has been variously defined in economic jargon, but the most widely accepted definition is the one by the World Bank – "access by all people at all times to enough food for an active, healthy life". Likewise, the World Food Summit at Rome in 1996 also known as Rome

Declaration on World Food Security on food plan action observed that, "Food security at the individual, household, national and global level exists where all people at all times have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life". In both definitions, emphasis has been given to physical availability and economic accessibility of food to the people. The mutant varieties are often grown by farmers in their fields, and any increase of food production resulted from the cultivation of the mutant varieties could be translated into increased food security, since this should be accessible for the people in need.

Physical availability and economic accessibility of food are the most important criteria of food security. Induced mutations have played a great role in increasing world food security, since new food crop varieties embedded with various induced mutations have contributed to the significant increase of crop production at locations people could directly access. An exact estimate of the area covered by commercially released mutant cultivars in a large number of countries is not readily available, but the limited information gathered clearly indicates that they have played a very significant role in solving food and nutritional security problems in many countries (Kharkwal and Shu 2009)

TURNING PLANT MUTATION BREEDING INTO A NEW ERA: MOLECULAR MUTATION BREEDING

Advances in molecular genetics and DNA technologies have brought plant breeding, including mutation breeding, into a molecular era. With ever-increasing knowledge of molecular genetics and genomics and rapidly emerging molecular techniques, breeders can now use mutation techniques in breeding new varieties more widely and efficiently than ever before. Plant molecular mutation breeding is here defined as mutation breeding, in which molecular or genomic information and tools are used in the development of breeding strategies, screening, selection and verification of induced mutants, and in the utilization of mutated genes in the breeding process. It is built upon the science of DNA damage, repair and mutagenesis, plant molecular genetics and genomics of important agronomic traits as well as induced mutations. Mutagenic treatment, super-mutable genetic lines, molecular markers and high throughput DNA technologies for mutation screening are the key techniques and resources in molecular mutation breeding and in increasing both the efficiency and efficacy of mutation techniques in crop breeding (Shu 2009).

Identification of mutants

Induced mutation and natural nucleotide variation are powerful tools for probing gene function and improving traits in plants. A major bottleneck in the routine application of induced mutagenesis to both crop improvement and genomic research 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.

In fact, mutagens such as ethyl methanesulphonate (EMS) cause stable point mutations and thus produce an allelic series of truncation and missense changes that can provide a range of phenotypes.

Traditional mutagenesis has been widely used in forward genetic strategies: identification of phenotypic variation and its association with gene changing.

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. In fact, TILLING (Targeting Induced Local Lesions IN Genomes) uses traditional mutagenesis and nucleotide polymorphism discovery methods for a reverse genetic strategy that is high in throughput, low in cost, and applicable to most organisms. In less than a decade, TILLING has moved from a proof of concept to a well-accepted reverse genetic method that has been applied to over 20 different species. Large-scale TILLING services have delivered thousands of induced mutations to the international research community. Advancements in new mutation discovery techniques promise to increase further the efficiency and applicability of the TILLING method (Till *et al*. 2009).

Mutants: phenotypic expression

A 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. Another major difficulty is the inherent problem of chimeras, a problem that is exacerbated in vegetative 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.

DOUBLED HAPLOID PRODUCTION

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. 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. 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. 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 boilogy techniques have resulted in the availability of reproducible DH protocols for over 250 plant species covering most plant genera (Forster *et al.* 2007).

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.

Below, recent findings in crop improvement promoted by advanced mutation approaches are described.

1) Sunflower mutants with improved growth and metal accumulation

Over the last two decades, the use of plants has been proposed as an alternative technique to remove toxic metals from contaminated soils. This technique, called phytoextraction, can use either hyperaccumulating species, able to accumulate and tolerate high amounts of metal, but producing low biomass, or high-yielding crops compensating moderate metal accumulation by a high biomass. Both types of plants can be considered for metal removal, but soil decontamination still takes quite a long time. Therefore, plants used for metal removal need to be improved for phytoextraction by chemical mutagenesis.

Improved yield and metal accumulation in sunflower mutants were already observed in the M_2 mutant generation, where three new sunflower phenotypes were found: mutants with a significantly enhanced biomass production and no changed metal accumulation; mutants with a slightly improved biomass production and an enhanced metal accumulation in shoots; and mutants with reduced metal uptake. The same alterations in growth and metal accumulation were observed in the following generation. The best M_3 sunflower mutants showed a three to five times higher cadmium, a four to five times higher zinc, and a three to five times higher lead extraction, as compared to the control inbred line. The stability of improved traits, yield and metal uptake, was confirmed also in the fourth generation, where mutant lines still provided a significantly enhanced metal extraction.

Metal translocation from root to shoot and distribution within the shoot (stem, leaves and flower) of mutant lines and control sunflowers grown on a metal contaminated soil was studied in detail in the fifth generation under greenhouse conditions. Sunflower mutant seedlings show a very good metal translocation capacity after three months of cultivation on contaminated soils; thus the metals were primarily accumulated by sunflower leaves (Nehnevajova *et al.* 2009).

2) Mutants pave the way to wheat and barley for celiac patients and dietary health

Wheat has two major nutritional problems for the consumer: (1) The flour or pasta produced from the grain is not acceptable to congenital celiac patients and may induce intolerance of dietary "gluten" in people later in life. (2) The grain is highly deficient in the essential amino acid lysine. Currently there is only one treatment for sufferers of celiac disease: the complete exclusion of wheat, barley and rye grains from their diets. Celiac disease is caused by an autoimmune reaction against undigested proline/glutamine rich peptides (epitopes) that are taken up through the intestinal mucosa and initiate an autoimmune response in human leucocyte antigen DQ2- or DQ8-positive individuals. This leads to chronic erasure of the microvilli of the intestinal epithelium and to permanent intolerance of dietary "gluten". Cereal prolamins are of two types: high molecular weight glutenins (HMWG) with a molecular structure of elastic fibrils that form dityrosine cross-links during dough formation and baking, and gliadins. The gene promoters of the gliadin-type proteins are silenced by DNA ethylation in vegetative tissues. This methylation is removed during grain development to permit protein synthesis. Inhibition of the demethylation by mutation specifically inhibits the synthesis of the gliadin-type proteins and only proteins consisting of elastic fibrils are produced. As a proof of principle, a barley cultivar called Lysiba already exists that has such a mutation and provides the rationale for creating wheat varieties by mutation of the 5-methylcytosine deglycosylases in the endosperm. Celiac patients are sensitive to a wide variety of different epitopes, which are located in the gliadin-type prolamins. Gliadin-type prolamins are of no importance for baking because wheat HMW glutenin has been shown to be alone sufficient to produce high quality breads (von Wettstein 2009).

3) Enhancing drought and salinity tolerance in wheat crop

Drought and salinity are major constraints on crop production and food security, and adversely affect entire countries over several years resulting in serious social, economic, and environmental costs. Water is in an extremely short supply in up to 10 eastern and southern Mediterranean countries. Wheat production in the Mediterranean region is limited mainly by the availability of water resources. Investigating the mechanisms by which wheat physiologically adapts to water deficits points to a salinity tolerance strategy showed that varieties of wheat which are able to maintain photosynthesis and growth at low soil Ψw often display a relatively greater capacity for leaf osmotic adjustment. Understanding the molecular basis of salt-stress signalling and tolerance mechanisms in wheat is required for engineering local wheat genotypes more tolerant to salt stress. This goal can be achieved only by first deciphering the physiological responses of wheat to salt stress. Recent work at the molecular level has led to the identification and cloning of cDNAs encoding proteins which are involved in the cellular-level physiological system which facilitates this adaptive response. Transgenic *Arabidopsis* plants overexpressing wheat candidate genes encoding ion transport proteins (TNHX1, SOS1, TVP1), or dehydrin (DHN-5) are much more resistant to high concentrations of NaCl and to water deprivation than the wild-type strains. Over-expression of the isolated genes from wheat in *Arabidopsis thaliana* plants is worthwhile to elucidate the contribution of these proteins in the tolerance mechanism to salt and drought. Testing candidate genes in TILLING available wheat population will allow the identification of new alleles conferring abiotic stress tolerance (Masmoudi *et al*. 2009).

4) Developing herbicide-tolerant crops from mutations

Herbicide-tolerant crops in combination with their corresponding herbicides are able to control many weeds that cannot be or are less effectively controlled with other means (Tan *et al*. 2005). Commercial herbicide-tolerant crops developed from herbicide-tolerant mutants include imidazolinone-tolerant maize, rice, wheat, oilseed rape, sunflower, and lentil; sulfonylurea tolerant soybean and sunflower; cyclohexanedione-tolerant maize; and triazine-tolerant oilseed rape (Duke 2005).

Most of the herbicide-tolerant mutants were developed through chemical mutagenesis followed by herbicide selection. Among the chemical mutagens, EMS was the most popular one. Several herbicide-tolerant mutants were also discovered through direct herbicide selection of spontaneous mutations. Although gamma irradiation was also attempted in mutagenesis for herbicide tolerance, no commercial herbicide-tolerance trait has been developed by using this method.

All mutations used in commercial herbicide-tolerant crops are derived from a single nucleotide substitution of genes that encode enzymes or proteins targeted by herbicides. Imidazolinone-tolerant maize, rice, wheat, and oilseed rape have a gene variant encoding an altered acetohydoxyacid synthase (AHAS) with the S653N amino acid

substitution. Additionally, imidazolinone-tolerant maize and oilseed rape have an AHAS with the W574L amino acid substitution. Imidazolinone tolerant sunflower has been developed from the A205V AHAS gene mutation. In contrast, sulfonylurea-tolerant sunflower selected from a farm field has an AHAS enzyme variant with the P197L amino acid substitution. Similarly, sulfonylurea-tolerant soybean has a P197S AHAS gene mutation (Green 2007). Sulfonylurea-tolerant sunflower from seed mutagenesis and imidazolinone-tolerant lentil are also derived from AHAS gene mutations. Cyclohexanedione-tolerant maize has an altered acetyl-CoA carboxylase with the I1781L amino acid substitution. Triazine-tolerant oil seed rape possesses a psbA gene variant that encodes the D1 protein of photosynthesis with the S264G amino acid substitution.

To confer commercial tolerance to herbicides, some herbicide-tolerance alleles can be heterozygous, others need to be homozygous, and the rest must be stacked with another tolerance gene. The alleles of all commercial herbicide-tolerant mutations are incompletely-dominant and not pleiotropic except for the triazine-tolerant mutation which is inherited maternally and linked with several agronomic traits. The herbicide tolerant trait can be incorporated in elite varieties through crossing of the elite variety with a trait donor.

5) Genetic enhancement of lentil (*Lens culinaris* Medikus) for drought tolerance through induced mutations

An attempt has been made to isolate a number of drought-tolerant mutants from four lentil cultivars by treating the seeds with physical (10, 20 and 30 kR of γ-rays) and chemical mutagens (0.04M of ethyl methane sulfonate and 0.05M of sodium azide) separately and in various combinations. The experiment was initiated during the winter season of 1999-2000 and carried over to advanced generations. The selection of environment (water stress or non-stress) for the development of drought-resistant varieties still remains controversial, however, the findings from present study suggest that materials ought to be tested in both stress and non-stress conditions so that the favourable alleles under drought can be maintained as well as selection response under favourable condition can be maximized. Yield under drought (Yd), yield potential (Yp), drought susceptibility index (S) and geometric mean (GM) were considered as the potential indicators for drought resistance of a family. Correlation coefficients between these parameters were calculated for selecting the parameter(s) which are more effective than others for screening the drought-resistant mutant line(s). It was observed that GM was positively and significantly correlated with both Yd and Yp, whereas it was negatively, but insignificantly correlated with S. There was significant, but negative correlation between S and Yd, while no significant correlation between S and Yp was observed.

From the correlation studies it may be concluded that for the enhancement of yield potential under both the conditions, selection should be based on GM rather than on S. Because S is a better measure of drought tolerance than a measure of performance under stress, genotypes may be first selected on the basis of high GM and then on the basis of high yield under drought (Yd). Twenty mutants lines selected on the basis of higher GM than their respective control, and were further evaluated for their yield performance under rainfed conditions and were subjected to drought tolerance tests through M4 to M6 generations. Three chemical tests, viz., nitrate reductase (NR) activity, protein content, and wax content were conducted and data were recorded on grain yield/plant. Nitrate reductase activity and wax content of most of the mutant lines were higher than their respective control and both were positively associated with grain yield, while protein content was lower in the mutant lines and was negatively associated with grain yield in that comparison. The lines showing higher nitrate reductase activity, wax content and grain yield appeared to be promising (Lal and Tomer 2009).

PERSPECTIVES

World food security deteriorated very sharply in the 1960's when developing countries like India, Pakistan, and Indonesia were desperately short of food grains. Fortunately, agricultural scientists responded with a new production technology, which has popularly been described as "Green Revolution Technology." This helped to avoid large-scale starvation for around 40 years. However, the food security problem has again seen a major deterioration in the last few years; food prices are rising sharply and once again the poor people of the world are threatened with serious malnutrition. The underlining causes that drove to food security deterioration, i.e. rising fuel and fertilizer prices, climate change related erratic rain falls, sudden and severe drought conditions, excessive floods, divert of food grains into bio-fuel production will remain for the years to come. Food security will even get worse since population is still growing while no significant expansion of arable lands is foreseen. FAO estimates that world food production should increase by more than 75% in the next 30 years to feed about eight billion people by 2025.

Therefore, a new "Green Revolution" is desperately needed to solve the food security issue in the years to come. The massive advent of plant molecular biology is anticipated to provide a sound solution to further increase food production by both increasing yield potential and stability. In this regard, induced mutagenesis is gaining importance in plant molecular biology as a tool to identify and isolate genes, and to study their structure and function. Recently, mutation techniques have also been integrated with other molecular technologies, such as molecular marker techniques or high throughput mutation screening techniques; mutation techniques are becoming more powerful and effective in breeding crop varieties. Mutation breeding is entering into a new era: molecular mutation breeding. Therefore, induced mutations will continue to play a significant role for improving world food security in the coming years and decades.

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-FLXF™ and Applied Biosystems SOLiDF™ 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.

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