Invited Article

New Facets of 21st Century Plant Breeding

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If an analogy of a duplex molecule like DNA is taken to be the backbone of agriculture, plant breeding forms one strand, agronomy/ technology being the other. Plant breeding includes the art and science for manipulating genetic systems to develop superior cultivars. Adaptation was the main trait used in selection, which was based on the model, or ideal, of the breeder. Primary methods of selection and the information available to plant breeders are selection based on phenotypes, breeding values, and genotypes.

Evolution of plant breeding

Plant breeding has been one of the longest, continuous activities carried out by humans. The evolution of the human civilizations paralleled the successes of plant breeding. The early plant breeders were effective in developing productive cultivated plants from wild species that were lower in productivity but possessed many important traits for their survival in nature.

The concept of natural selection by Darwin in 1859 followed by the rediscovery of Mendel's laws of genetics in 1900 and their integration provided the foundations of modern plant breeding.

The landscape of plant breeding has changed dramatically during the past 100 years. The relative importance of art vs. science in plant breeding also has changed with greater emphasis on science. The human and financial resources allocated to plant breeding research have had significant changes during the last half of the 20th century (Frey, 1996). By year 2000, plant breeding had been transposed from primarily the public sector to the private sector. In contrast, resources allocated to plant breeding in the public sector either significantly decreased or diverted to the study of molecular genetics. Thus, today's ten largest agro-tech companies have a global share in seed sales of more than 60% with trade interest largely in hybrid seeds of leading crops as they provide a solid return on investment.

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The concept of using first generation hybrid as direct crop cultivar especially in cross pollinated crops impacted research during the 20th century and in1930's and 1940's the inbred-hybrid concept was extensively tested and hybrids were rapidly accepted by the farmers.

Rapid progress also has been made in many non-genetic areas, such as plot equipment, computer systems for recording field data, field designs, statistical analyses, defining target environments, etc. These have contributed significantly to increasing the efficiency of plant and progeny selection, or determining the breeding values for data taken at the phenotypic level (Hallauer and Pandey, 2006).

Phenotype, Genotype and Breeding Value

Phenotypic selection is certainly the method of choice with sustained and continuous use in plant improvement. The long term effects of phenotypic selection led to productive crop species and varieties.

Phenotypic selection obviously has been more important in developing our current germplasm resources and probably can be regarded as one of plant breeding's greatest accomplishments (Hallauer, 2011). Phenotypic selection, therefore, was very effective for many traits but not so for grain yield.

The basic unit of selection, either natural or human, has been the genotype. During the millennia that included domestication, phenotypic selection, evaluation of breeding values, and presently when

molecular genetics is employed in the choice of parental genotypes, and in development of genetically modified organisms (GMOs), the genotype of individuals remains the unit of selection (Hallauer, 2011).

Vilmorin in France was the first to suggest and use the progeny test in 1859 for the improvement. The concept of determining the breeding values of parents via progeny testing was one of the major factors for productivity advances made during the 20th century. Most studies suggest that 50 to 60 % of the advances made in maize grain yields were because genetic improvements made in the inbred lines and hybrids.

To be proficient in identifying superior cultivars, the breeder has to accurately determine the relative breeding values of the progenies that are being evaluated. Since the introductions of the concepts of experimental design and statistical analyses by Fisher (1925), researchers have continued to enhance and refine the experimental design and statistical analyses to reduce experimental error and increase the precision of estimates of breeding values. Breeding values will continue to be an important component in future plant breeding programs - in choice of parents for developing breeding populations.

Green Revolution and Food security

Genetic improvement coupled with better crop management triggered the Green Revolution in the 1960s leading to dramatic improvements in yield, particularly under irrigation in developing countries, and in turn to food surpluses and to cheaper food in the developed world (Hall and Richards, 2012). Yields of our major crop species have gradually increased during the past 100 years. The twentieth century witnessed an incredible increase in wheat grain yields, from little more than 1 t / ha to more than 10 t / ha today under good farming conditions (Figure 1). We need to sustain these efforts to feed the predicted 9 billion humans inhabiting the earth by 2050 or nearly 37 % more inhabitants in 2050, compared with 2010 (Hallauer, 2011).

As India grapples with the nuances of a proposed food security law, an UN body has reported that the global Green Revolution of the 60s that had increased agricultural productivity in parts of the country is now fast losing momentum. “The Green Revolution (has) started to run out of steam” (Nandan, 2012).

To achieve global food security by 2050 primary production must almost be doubled, at least to 80 % by increasing production per unit land. Annual breeding progress must be at least 1.75% compared to the current rate of 1.4%, if we are to meet the target of doubling yields by 2050 (GHI, 2010; Figure 2).

Deceleration in agriculture growth

Growth rate of agriculture GDP has slipped from 3.62% during the period1984-85 to 1995-96 to less than 2% in the period between 1995-96 and 2004-05. Per capita food grains production is now at 1970s levels (Planning commission, 2007; Figure 3). Evidence of large gaps between what can be attained at the farmer’s field with adoption of technology as compared to what is obtained with existing practices are depicted in Figure 4. Yield gaps vary considerably from crop-to-crop and from region-to-region. But, a look at the yield gap mentioned above conveys that there is a large potential for raising output with effective dissemination of existing technology.

Technology and plant breeding fatigue?

Analysis of new varieties released of major crops (rice, wheat, maize, groundnut, mustard and sugarcane) shows significant deceleration of the growth of yield potential, with negligible increase over the last decade (Figure 5). This technology fatigue has to be addressed urgently by changing research priorities suitably. The genetic reason for lack of increase of farm yields may be that the major target traits have been fully exploited.

Historically**,** hybridization and induced mutagenesis are the means for creating variation and hybrid technology to harness heterosis and for creating recombinants with improved yield and biotic and

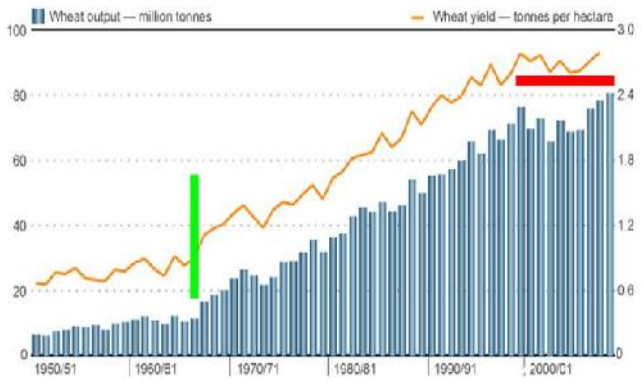

Figure 1. Indian wheat production and productivity between 1950 and 2010 (Morel, 2010)

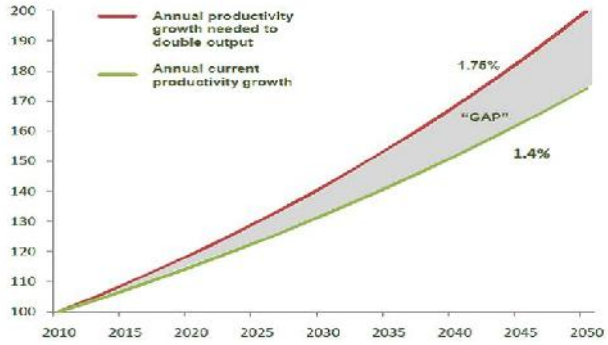

Figure 2. Gap between demanded and actual annual productivity rates for doubling the current grains output globally (GHI, 2010)

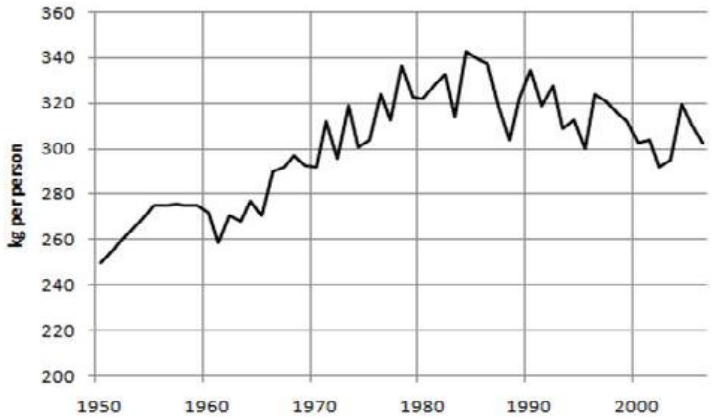

Figure 3. Per capita food grain production between 1950 to 2006 (Planning Commission, 2007)

Crop	Yield gap (%)	State
Wheat	6	Punjab
	84	MP
Maize	7	Gujrat
	300	Assam
Jowar	13	MP
	200	Karnataka
Rice	> 100	Assam, Bihar, Chattisgarh, UP
Soybean	7	Rajasthan
	185	Karnataka
Mustard	5	Haryana
	150	Chattisgarh
Sugarcane	16	AP
	167	MP

Figure 4. Yield gaps observed in some crops across different states of India (Planning Commission, 2007)

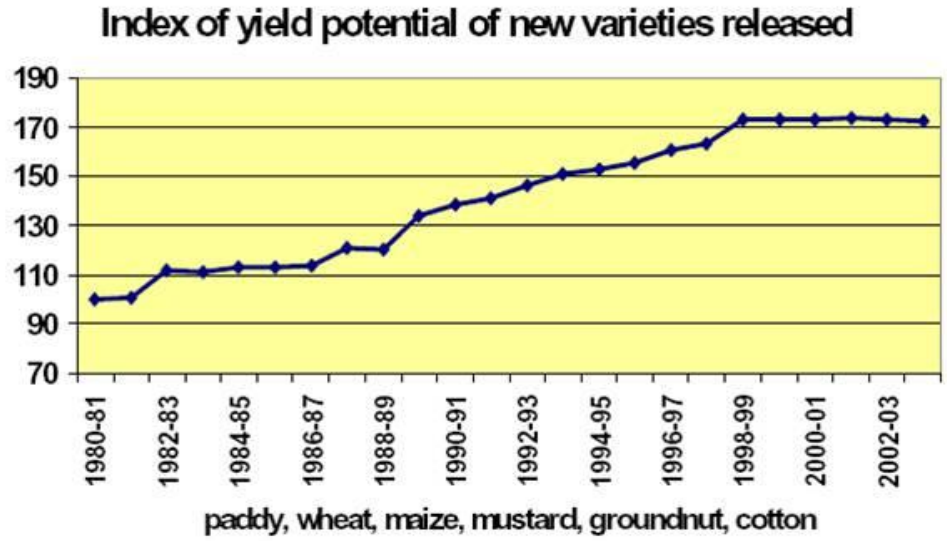

Figure 5. Index of yield potentials of new varieties released in last two decades (Planning Commission, 2007)

abiotic stress resistance. But the pace and precision of these techniques/ procedures are low, thereby dragging down the conventional breeding approaches and also resource use efficiency.

With the advent of PPV-FR and NBA regimes in India, the inflow of new variability through exotic as well as indigenous germplasm has dwindled to a trickling; thereby making the technologies that can generate desired variability in precisely targeted and/or controlled ways the new 'efficiency boosters' in breeding programs.

Can we speed up breeding progress?

If tools and techniques form new biology will have to greatly accelerate the breeding process, they have to be integrated intelligently to meet faster the challenges to food security (Lusser *et al*., 2011). Marker-assisted selection (MAS), is being implemented to assist breeders in achieving their goals as quickly as possible, revealing the best allele combinations, even for polygenic traits. The breakthrough is partly due to modern DNA-chip technology, by which thousands of markers can be analyzed economically in one step. Physiology and biochemistry are now better understood at the genetic level contributing to more reliable phenotyping.

As new, well-tested technologies emerge, especially molecular tools like MAS, plant breeding will become more precise and fast. This should principally speedup breeding progress, starting with the more efficient use of genetic resources to a reduction of multi-location trials as genotypic selection improves in the near future. Gene technology can additionally solve problems related to quality and resistance within and across the species borders, crucial timesaving to maintain the vigor of global crops and to provide better starts for underutilized crops. We must surpass the present outcome of one variety from 100,000 seeds to five good varieties from even fewer seeds in the near future (Stamp and Visser, 2012).

Synergy of plant breeding and biotechnologies

Genetic modification has been the basis for betterment of crops in modern agriculture. With the advent of principles of genetics, plant breeding has become a science based technology contributing to directed evolution of crop varieties. If the benefits of the information derived from molecular genetic studies are to be utilized, the biotechnology tools become a necessary component of the matrix for the potential use of information and products derived from the molecular genetics.

The past century has witnessed breath taking array of discoveries in biological sciences in general and in molecular biology in particular leading to deployment of various tools and techniques to broaden the possibilities for breeding new crop varieties. Tissue culture, micro-propagation, haploids, embryo rescue and protoplast fusion greatly facilitated rapid generation of uniform plants and obtaining hybrids between incompatible species. Further innovations in plant breeding came from the introduction of DNA based technologies and insight into the genome - sequence information and its functional relatedness. Two such important technologies widely used are: i) DNA markers for identification, tracking of targeted gene or selection of favourable combination of genes (DNA marker technology); and ii) Introgression of alien genes through genetic engineering (Recombinant DNA technology). The capability to transport the genes from secondary, tertiary and quaternary gene pools via transgenesis has brought a new dawn in plant breeding, enabling breeders' access to here-though inaccessible genes/ gene-pools. Marker technology facilitated breeders to exploit the genetic variation within the crop gene pool while genetic engineering provided access to biodiversity as a whole (Figure 6).

Speedy derivation of inbred lines and generation of novel recombinants is the goal of all plant breeding activities. Rapid generation advance (RGA) and doubled haploids (DH) through- anther culture (rice), inducer stock (maize) or bulbosum technique (barley) have been successfully deployed for this purpose.

Doubled haploids

Production of haploid plants that inherit chromosomes from only one parent can greatly accelerate plant breeding. Haploids generated from a heterozygous individual and converted to diploid create instant homozygous lines, bypassing generations of inbreeding. Several methods are

generally used to produce haploids. First, cultured gametophyte cells may be regenerated into haploid plants but many species and genotypes are recalcitrant to this process. Second, haploids can be induced from rare inter-specific crosses, in which one parental genome is eliminated after fertilization. Ravi and Chan (2010) showed that haploids can be generated through seeds by manipulating a single centromere specific histone protein CENH3 coded by *cenh3* gene. The *cenh3*-null mutants when crossed with wild type, chromosomes of null mutants are eliminated, producing haploid progeny. Haploids are spontaneously converted into fertile diploids through meiotic non-reduction, allowing their genotype to be perpetuated. Maternal and paternal haploids can be generated through reciprocal crosses. This process has key advantages over current methods for producing haploid plants. First, no tissue culture is needed, removing a major source of genotype dependence. Second, the same inducer produces maternal and paternal haploids. Third, crossing a *cenh3* mutant as the female transfers the nuclear genome of the male parent into a heterologous cytoplasm. This could accelerate production of cytoplasmic male sterile lines for making hybrid seed. Fourth, genome elimination occurs between parents that are isogenic except for CENH3 alterations, avoiding fertility barriers inherent to wide crosses.

With the discovery of an array of DNA markers (RFLP, RAPD, AFLP, SSR and SNPs) and the potential benefits of using DNA markers linked to gene(s) of interest in breeding, paved the way to move from phenotype to genotype-based selection. Association mapping, genomic selection (GS) and genome wide association study (GWAS) are the new marker based techniques enlarging the canvas of selections in plant breeding.

Marker assisted selection (MAS) will enhance selection for more complex traits (e.g., yield, drought tolerance, tolerance to acid soils, disease resistance, quality, etc), but the one major difficulty will be the resolution of gene interactions and their interactions with environments; these are the same problems that have impacted plant breeding throughout its long history. Information from molecular genetics has enhanced plant breeding by identifying the appropriate parents to include in breeding crosses, assignment of genotypes to appropriate heterotic groups, and major alleles that will enhance selection for complex traits. Genome wide association (GWA) mapping which looks for associations between phenotypes of interest and the DNA sequence variations present in an individual's genome, as assessed by determining individual's genotype at positions of hundreds of thousands of single nucleotide polymorphisms (SNPs). GWA studies provide much higher resolution than linkage mapping because they involve studying a natural population rather than the off-spring of crosses, and associations in natural populations are typically on a much finer scale because they reflect historical recombination events. With the combination of GWA studies and forward-genetic approaches, it will finally become possible to bridge the genotype-phenotype divide (Nordborg and Weigel, 2008).

The gene rich regions (GRRs) of the genomes are being identified to bring down the sequencing work to a manageable level. This will also help in the discovery of new genes, allele-mining, and large scale SNP genotyping. The future plant genomics research will certainly derive benefit from the recent development of new-generation sequencing (NGS) technologies, including Helicos true single molecule sequencing (tSMS) technology (Gupta and Xu, 2008).

The NGS technologies certainly help plant genomics research in a variety of ways. While more plant genomes will be sequenced, epigenomes, transcriptomes, and metabolomes will also be worked out with much higher speed and at a cost reduced by several orders in magnitude. The science of plant genomics will be further amplified by the emerging areas of "chemogenomics" and "synthetic genomics."

Functional genomics is another area that received more attention, and some issues addressed significantly include gene isolation through map-based cloning and candidate gene approach, as well as functional analysis through insertional mutagenesis, TILLING, transcription profiling, RNAi, etc.

TILLING (for Targeting Local Lesions IN Genomes) searches the genomes of mutagenized organisms for mutations in a chosen gene, typically single base-pair substitutions. TILLING provides a range of mutant alleles and is potentially applicable to any organism that can be effectively mutagenized. TILLING extends the long-established practice of using existing variation for functional genetic discovery (Comai and Henikoff, 2006).

Computer software and hardware advances have made possible the greater incorporation of genetic information that can be used in selection. The increased information available has dramatically increased the relative importance of science, compared with art, in plant breeding.

Handa *et al*. (2010) have suggested some of the avenues for fruitful fusion of breeding and biotechnologies. Sreenivasulu *et al*. (2010) have indicated the way in which the interactions between structural and physiological/ functional streams can lead to the integrative genomics for knowledge enabled breeding. In the model, genetic maps provide the inputs needed through functional markers, association mapping, expression genetics, MAS and genomics assisted breeding, while transcriptome analysis provide the inputs from transcriptional networks, gain and loss of function, kinetic models. Collard *et al*. (2008) and Fakrudin *et al*. (2012) have reviewed the synergistic scenarios in depth and are very enthusiastic about the exciting scenario unfolding in the field of omics enabled designer breeding. With the help of systems biology, these are interleaved to attain the synergistic integrations to achieve knowledge driven breeding (Fakrudin et al., 2012; Figure 7).

Optimistically, it seems further genetic progress can be sustained because as greater genetic information at the molecular level is understood and integrated with phenotypic selection, increasing the effectiveness of selection. It is essential that increased crop yields on per unit area be sustained in the future. New generation of plant breeders with education and training in molecular genetics will have greater participation in the breeding methods, particularly for the improvement of complex traits.

New generation plant breeding techniques

Innovation in plant breeding is necessary to meet the challenges of population growth and climate change. Agriculture has been able to cope with these challenges until now. However, further efforts are needed and therefore plant breeder's quest for new plant breeding techniques (Lusser *et al*., 2011).

Like transgenosis, some of the new plant breeding techniques such as cisgenesis/ intragenesis and floral dip, a variant of agro-infiltration aim to achieve the stable insertion of a new gene. The grafting of non-GM scions on GM rootstocks results in chimeric plants where only the lower part carries the genetic transformation.

In the case of most of the other techniques (e.g. ZFN-1 and -2, reverse breeding, agro-infiltration "*sensu stricto*" agro-inoculation and RNA directed DNA methylation, i.e., RdDM), a new gene is delivered to the plant cells in an initial step. However, this gene is only transiently expressed in the target cell or stably integrated in an intermediary. By sifting the progeny of the transformed plants, and (if necessary) segregating out the offspring that carry the inserted gene, transgene-free crops are achieved. ZFN-1 and -2 and oligonucleotide directed mutagenesis (ODM) aim to accomplish targeted mutagenesis (changes of one or a few base pairs). The application of RdDM results in the methylation of the promoter of the target gene which is consequently silenced. In the case of reverse breeding (which is used to reconstitute elite parent plants), agro-infiltration "*sensu stricto*" and agro-inoculation (which are applied for the selection of the most suitable plants) no stable changes in the genome of the commercialized crop are intended.

These techniques have been adopted by commercial breeders and the most advanced crops could reach the stage of commercialization in the short to medium term (2-3 years) in the event of these techniques not being classified as resulting in GMOs. The techniques show great technical potential, but efficiency still has to be improved. The main constraints for the adoption of the techniques are the regulatory uncertainty and the potentially high costs for risk assessment and registration (if the crops derived by these techniques are classified as GMOs).

Crops resulting from most of the techniques cannot be distinguished from conventionally bred crops and detection is therefore not possible.

Zinc finger nuclease (ZFN) technology

ZFNs are proteins custom-designed to cut at specific DNA sequences. They consist of a "zinc finger" domain (recognizing specific DNA sequences in the genome of the plant) and a nuclease that cuts double stranded DNA.

ZFN-1:

With the ZFN-1 approach, no repair template is provided to the cells together with the ZFN proteins. The DSB is corrected by non-homologous end-joining (NHEJ), which is a natural DNA repair system in the cell. This often results in substitutions to one or only a few bases or in small localized deletions or insertions. The ZFN-1 technique can therefore be used as an efficient mutagenesis method. When these mutations occur in coding regions, they may produce a frame shift, a deletion of one or more amino acids or changes in amino acids, thereby resulting in a high frequency of gene knock-outs.

ZFN-2:

With the ZFN-2 approach, a continuous stretch of DNA is delivered to the cell simultaneously with the ZFN. This template DNA is homologous to the targeted area, spanning a few kb, and overlaps the region of the double strand break (DSB). The template DNA contains the specific base pair alterations to be introduced in the genome by homologous recombination (HR), which occurs at a very low rate in plants compared to NHEJ. The application of the ZFN-2 technique therefore allows the increase of the number of mutations targeted to a certain locus in the gene and the introduction of the base pair(s) of choice compared to random mutations.

ZFN-3:

With the ZFN-3 approach a recombinant DNA molecule is constructed in which the DNA fragment of the gene cassette of interest is sandwiched between stretches of DNA that are homologous with the DNA sequences flanking the DSB site. This DNA construct, together with the ZFN, is delivered to the cell. Transgene integration targeted to an endogenous genomic locus in the cell can be obtained by HR.

The current state-of-art of the technology, nonspecific mutations resulting from non-specific binding of the ZFNs are likely to occur leading consequently to unintended mutations. Four-finger ZFNs that recognize 24 bp DNA sequences have been shown to promote highly sequence-specific cleavage in human cells. It is therefore hypothesized that four-finger ZFNs would increase specificity compared to three-finger ZFNs. A point to consider for safety is that with the ZFN technique multiple subsequent site-specific changes may be induced in a single organism, which is not possible by chemical or natural means.

Currently ZFNs for approximately half of the 64 nucleotide triplets are available. ZFN libraries are being up-dated to improve genome coverage. It is also necessary to improve the specificity and efficiency of ZFNs. ZFNs are subject to an extensive selection and validation process before being commercialized. In parallel smart approaches for selection of the mutated plants have to be developed.

Oligonucleotide directed mutagenesis (ODM)

ODM employs oligonucleotides for the induction of targeted mutations in the plant genome. They target homologous DNA and induce site-specific nucleotide substitutions, insertions or deletions through repair mechanisms. If the oligonucleotides and the experimental protocol are adequately designed, the mutation induced by ODM should be highly specific. Organisms developed through ODM cannot be distinguished at the molecular level from organisms bearing the same mutation obtained through mutation techniques such as irradiation or chemical mutagenesis or through selection from natural populations. An advantage of this technology is that it does not use integrative vectors and thus eliminates the risk of any associated insertional mutagenesis. It also acts on specific genes and does not introduce foreign DNA sequences into the target genome. However, the mutation rates achieved are usually low and are comparable to the rate of spontaneous mutations. Potential safety issues (for crops obtained through any of these approaches) may be related to changes in the expression of endogenous genes or to a specific change in the amino acid sequence of an endogenous protein.

ODM has to be applied to protoplasts. This limits its application to certain crops and expertise for the production and regeneration of protoplasts has to be acquired. To achieve higher mutation efficiency, the design of the oligonucleotides has to be improved. Furthermore, methods for efficient screening of the mutated plants have to be developed.

Cisgenesis and Intragenesis

When applying the cisgenesis/intragenesis technology a DNA fragment from the plant species itself or from a cross-compatible plant species is inserted into the plant genome (Figure 8). In the case of cisgenesis, the inserted gene is unchanged and includes its own introns and regulatory sequences. In the case of intragenesis, the inserted DNA can be a new combination of DNA fragments from the species itself or from a cross-compatible species. Cisgenic and intragenic plants are produced by the same transformation techniques as transgenic plants, e.g. *Agrobacterium*-mediated transformation, following the isolation of genes from the host. Biolistics could also be used. The changes intended when applying this technique relate to modifying the expression of target genes through stable integration in the host genome, as is the case for transgenesis.

Specific vectors have been constructed for cisgenic/ intragenic approaches which use DNA sequences originating from the same crop species or related species to insert the target genes. These sequences have sufficient homology with *Agrobacterium* T-DNA sequences to allow this function. This approach is termed the P (plant)-DNA approach. Where P-DNA approaches are used, bacterial DNA is absent. Irrespective of whether the cisgenic or intragenic approach is used there exists a possibility that the inserts interrupt open reading frames (ORFs) in the host plant or create new ones as a consequence of the insertion process. Deletion of host DNA can also occur following insertion. This could give rise to unintended effects. The same issues are identified as a possible risk for transgenics, for mutation breeding and variation induced by somaclonal variation.

A possibility exists that inserts interrupt known ORFs (which may lead to gene silencing) or create new ones as a consequence of the insertion process (possibly leading to the production of new proteins). Deletion of host DNA can also occur following insertion. Conventional breeding can also result in disruptions to ORFs and other molecular changes including deletions and recombinations. The same can be said for mutation breeding and variation induced by somaclonal variation. The cisgenic/intragenic approach is based on the assumption of cross-compatibility of the host plant and the plant used to provide the genes.

Given that cisgenic/ intragenic organisms may contain new proteins, or greatly altered levels of familiar proteins, it has been argued that they generate similar concerns about safety as transgenic organisms. Cisgenesis/ intragenesis takes advantage of the experience gained in the use of transgenesis, a technology that in principle applies the same plant transformation methods. However, some problems related specifically to cisgenesis/ intragenesis still have to be addressed, such as the search for and isolation of suitable genes within the gene pool of the crops, investigation of the functioning of plant-derived promoters or the development of marker-free approaches.

RNA-dependent DNA methylation (RdDM)

When applying the RdDM technique, genes encoding RNAs which are homologous to plant sequences, like promoter regions, are delivered to the plant cells. These genes, once transcribed, give rise to the formation of small dsRNAs. They induce methylation of the homologous sequences and consequently inhibit their transcription. The efficiency of silencing can be up to 90% and is dependent on the active transcription of the promoter. Generally, the degree of silencing is related to the degree of methylation, but this is not always the case. Silencing and differences in silencing have been observed to be transmitted to at least the F_3 generation. Methylation is restricted to the region of sequence homology with the dsRNA. When the template RNA for dsRNA is introduced by transfection or by a vector system, the templates are intended to be present only transiently in the cell and are expected to be absent from the final commercialized product. It is not clear for how many generations the effect of gene silencing by RdDM remains in the absence of the inducing construct. An unintended effect could therefore be

the loss of silencing of the specific gene in the commercial product. Another potential unintended effect could be the silencing of genes with homologous promoter sequences. Potential safety issues may therefore only be related to changes in the expression levels of targeted endogenous genes.

The applicability of RdDM has to be investigated on more crop plants and the durability of the gene silencing in particular has to be investigated and improved. Furthermore the design of the transgene encoding dsRNA needs to be improved. Methylation is restricted to the region of sequence homology with the dsRNA. Therefore, it is necessary to investigate further the functioning of the promoters and especially to study which sequences are relevant for the regulation of gene expression.

Grafting on GM rootstock

Ground vegetative component of one plant (also known as the scion) is attached to a rooted lower component (also known as the rootstock), of another plant to produce a chimeric organism. With regard to plant breeding, the grafting of a non-GM scion onto a GM rootstock is considered to be the main approach. However, it is also possible to graft a GM scion onto a non-GM root stock and indeed a GM scion onto a GM rootstock. If only the rootstock is transformed then intended changes to the genome are targeted at root tissues. However, it is conceivable that there might be an intention to transform only the rootstock with a view to changing protein or gene expression in the scion due to the movement of specific proteins and/or RNA from the roots to the scion. In this way a GM rootstock could be used to introduce new traits into a range of genetically distinct scions.

With respect to the possible movement of DNA between rootstock and scion which could result in genome changes in the scion there is little evidence that this is an issue. Also the transfer of plastid genetic information in a graft from rootstock cells to the cells of the scion and vice versa has been reported. Genetic exchange appeared to be restricted to graft sites only. Therefore, one could conclude that unintended changes to the coding sequence of a non-GM scion grafted onto a GM rootstock do not occur.

In transmission of other macromolecules from root to scion, it is known that recombinant proteins, hormones and non-coding RNA (e.g. siRNAs [small interfering RNA]) can be transported from the GM rootstock of a graft to the scion where they can induce an effect. It is known that RNAi can lead to RNA-directed DNA methylation of promoter regions, resulting in modified expression of the target genes. The major issue relates to any unintended changes in gene, protein and trait expression in the scion resulting from unwanted movement of proteins and RNA from GM roots to non-GM scions. Grafting on GM rootstock combines two breeding techniques with a long history of use: grafting and genetic transformation. Though the technique is well developed, the influence of different rootstocks on the physical appearance of the scions is known, knowledge of the movement of molecules from the rootstock to the scion and their influence on gene expression in the scion need to be investigated further.

Reverse breeding

The intended goal of the reverse breeding technique is to generate perfectly complementing homozygous parental lines through a suppression of meiotic crossovers and the subsequent fixation of non-recombinant chromosomes in homozygous doubled haploid (DH) lines. In this respect, there are no changes foreseen in the genome of the selected non-GM offspring. Unintended effects could include the silencing of other homologous sequences in the genome as a result of the presence of the RNAi construct. This would not induce genomic changes, but could affect expression levels. Another unintended effect of the technique could be an incomplete suppression of meiosis (Figure 9).

Silencing of other homologous sequences in the genome by the RNAi construct could affect expression levels, which can also occur under natural conditions. Suppression of meiosis, incomplete or not, can also be obtained by chemical and physical means or by environmental factors (Moazed, 2009).

Reverse breeding is a very young technique and therefore research is still required to overcome technical problems and to fully exploit its potential. For example, research is being carried out to test

alternatives to transformation for obtaining the suppression of recombination, like Virus Induced Gene Silencing (VIGS), graft transmission of silencing molecules, knock-out mutations or the use of chemicals that repress crossover. The recent developments to manipulate the meiosis and crossing over promise to further enhance these capabilities to produce haploids by centromere heterochromatin (CNH3) mediated genome elimination. Additional research is needed to improve the efficiency of DH formation.

A further development related to meiotic crossing over is the identification of a key factor, an enzyme in the helicase family, limiting meiotic crossovers - The Fanconi Anemia Ortholog (*FANCM*) gene (Knoll et al., 2012), the manipulation which holds promise in plant breeding to tame the recombination process. A single mutation in this gene leading to three fold number of cross overs, without having any effect on the fertility or health of the plant (Crismani et al., 2012). This opens up a new window in increasing the genetic recombination, thereby providing opportunities of obtaining hitherto unknown combination of traits of interest.

Agro-infiltration

Depending on the tissues and the type of constructs infiltrated, three types of agro-infiltration can be distinguished:

1. "Agro-infiltration *sensu stricto*": Non-germ line tissues are infiltrated with a liquid suspension of *Agrobacterium sp.* containing a genetic construct in order to obtain localized expression in the infiltrated area.

2. "Agro-inoculation" or "agro-infection": Non-germ line tissues (typically leaf tissues) are infiltrated with a construct containing the foreign gene in a full-length virus vector in order to obtain expression in the entire plant.

3. "Floral dip": Germ line tissues (typically flowers) are infiltrated with a DNA construct in order to obtain transformation of some embryos that can be selected at the germination stage.

The intended goal of the technique is the transient and temporary expression of specific coding sequences without integration of the introduced DNA in the plant genome. However, in the case of the floral dip it the aim is to obtain stably transformed seedlings without the need for a plant cell regeneration phase. The resulting plant has the same properties as a transgenic plant.

While the aim is the transient and temporary expression of a coding sequence, the integration of T-DNA fragments into the genome of cells in the infiltrated area cannot be ruled out. In the case of agro-inoculation/ agro-infection, the spreading of the gene construct introduced into the viral genome is caused by systemic spreading of RNA viruses throughout the plant via plasmodesmata. Since the gene construct is spread via RNA molecules, they do not integrate into the plant genome.

The technique is well developed. However, to date it is only applied in a small number of plant species and tissues. Research into in the possible expansion of its applicability might be of interest in the future. Although only transient and local gene expression is intended, spreading and integration of *Agrobacterium* and integration of the T-DNA cannot be excluded. Further research is therefore required, including the testing for the presence of *Agrobacterium* and for the integration of T-DNA.

Synthetic chromosomes

Synthetic chromosomes provide the means to stack transgenes independently of the remainder of the genome. Telomere-mediated truncation coupled with the introduction of site-specific recombination cassettes has been used to produce mini-chromosomes (Gaeta et al., 2012; Figure 10). Synthetic chromosomes establish the means to add or subtract multiple transgenes, multigene complexes, or whole biochemical pathways to plants to change their properties for agricultural applications or to use plants as factories for the production of foreign proteins or metabolites. Production of artificial "mini-chromosomes", or gene stacks, that are designed to be inserted into a plant's genetic apparatus, to harness the plant's natural dynamics for the purposes of making a higher yield are in offing in biofuel programs such as that of Chromatin Inc., USA (Al

Fin Energy Blog, http://alfin2300.blogspot.in/ 2010/04/gcc-genetic-engineering-has-always-held.html). Combining these synthetic genomes with haploid breeding could provide the means to transfer many transgenes more easily among varieties of the same species (Gaeta et al., 2012).

Gene pyramiding and stacking is becoming popular and has already occupied more than 39% area under GM crops (Yu and Birchler, 2007). Currently, many generations of backcrossing are usually required to achieve this, avoiding the linkage drags. This has created a bottleneck that limits the advancement of plant genetic engineering technology. This bottleneck could be overcome by utilizing the engineered mini-chromosome system which allows for the expression of almost unlimited number of genes that can be inherited together as an independent genetic unit.

However, the epigenetic nature of centromere formation and remarkably variable molecular mechanisms of the functional centromeirc elements (Dhar and Kour, 2011) can complicate the production of synthetic chromosomes.

Additional new plant breeding techniques

Meganucleases are proteins that specifically recognize target DNA sequences of 12 to over 30 base pairs and create a double strand break (DSB) that activates repair mechanisms and DNA recombination (dePalma, 2010). Similarly to ZFNs, the technique can be used for site-specific mutagenesis or for targeted gene insertion by homologous recombination. Newly designed meganucleases can be produced in order to induce site-specific DNA recombination at a chosen locus in plant cell. The meganuclease technique is relatively advanced. Like ZFNs, meganucleases can be used for site-specific mutagenesis or for targeted gene insertion by homologous recombination.

Significant progress during the last two decades has been made in different areas of genomics research. These include development of thousands of molecular markers (including RFLPs, SSRs, AFLPs, SNPs, and DArT markers), construction of molecular genetic and physical maps (including radiation hybrid maps for some chromosomes) with reasonably high density of markers, development of more than 1 million ESTs and their use for developing functional markers, and the development of BAC/BIBAC resources for individual chromosomes and entire sub genomes to facilitate genome sequencing. Functional genomics approaches like TILLING, RNAi, and epigenetics have also been utilized successfully, and a number of genes/QTL have been cloned to be used in future crop improvement programs. Organellar genomes including chloroplast and mitochondrial genomes have been fully sequenced. The available molecular tools also facilitated a revisit of the crop improvement community for identification of markers associated with all major economic traits leading to the development of major marker-aided selection (MAS) programs for crop improvement in several countries.

The Seed Production Technology (SPT) process is an innovative and non-invasive approach to hybrid seed production that uses proprietary technology to prevent pollen shed by seed parents.

This dramatically reduces the risk of a seed parent pollinating itself, and ultimately improves the overall quality of the hybrid seed. Additionally, while the SPT Process uses a transgenic maintainer line at the front end, the progeny and resulting hybrid seed will not contain the SPT transgenes. The SPT is a process and not a product (Pioneer, 2012).

Are these technologies for real?

From patent search results it emerges that around 50 organizations are active in the field of new plant breeding techniques (Figure 11). Although private companies are leading in number of patents, the public sector is also active in patenting related to new plant breeding techniques. So, the availability of these new plant breeding techniques is shrouded by IPR regime, their existence and utilization is beyond doubt. In fact, the products from employing techniques are expected to hit commercial availability phase in next three to five years (Lusser et al., 2011).

With the vast panorama of possibilities available and emerging to manipulate the plant's genetic architecture dealt above, it is clear that the process of plant breeding continues to evolve from a

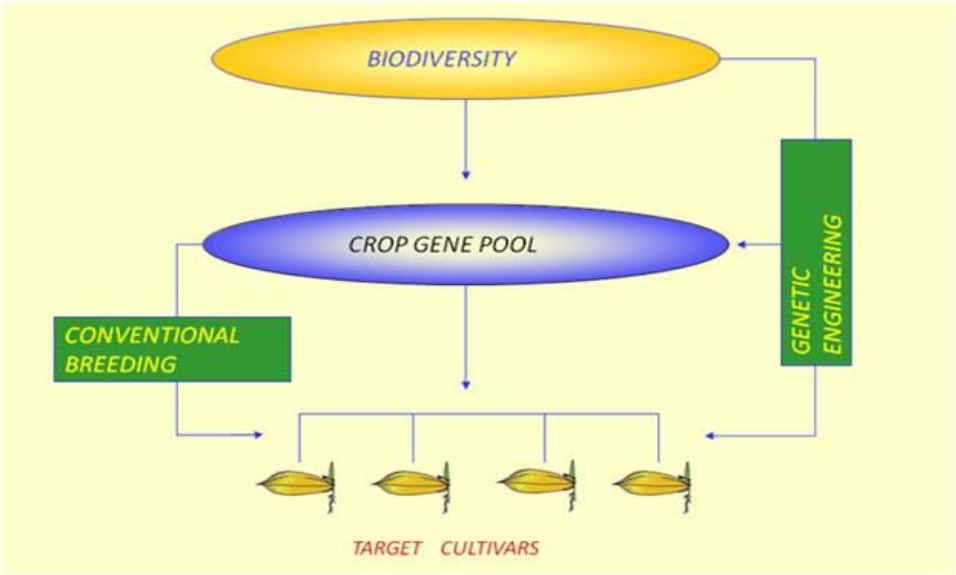

Figure 6. Expansion of gene pool accessibility and utility for plant breeding

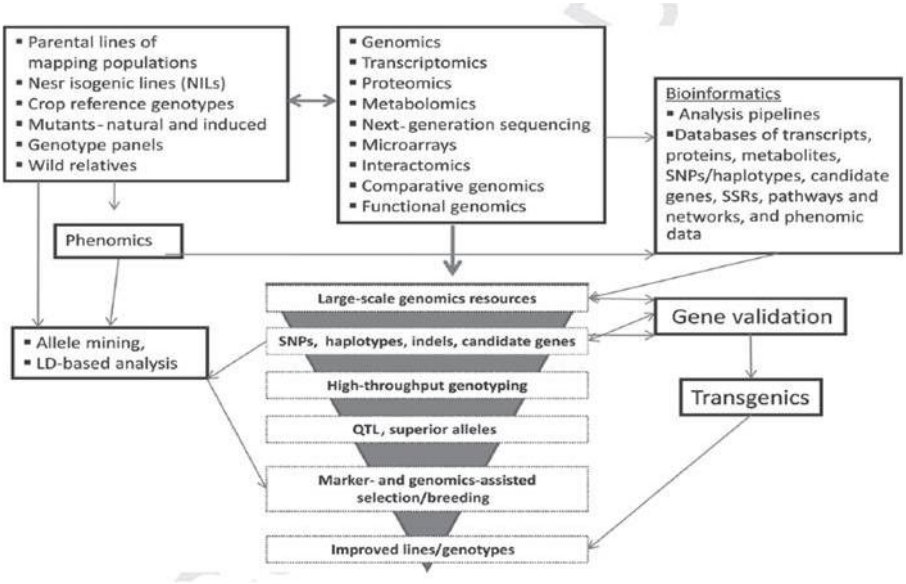

Figure 7. Interactomics: the integration of omics technologies and their utilization in crop research (Fakrudin et al., 2012)

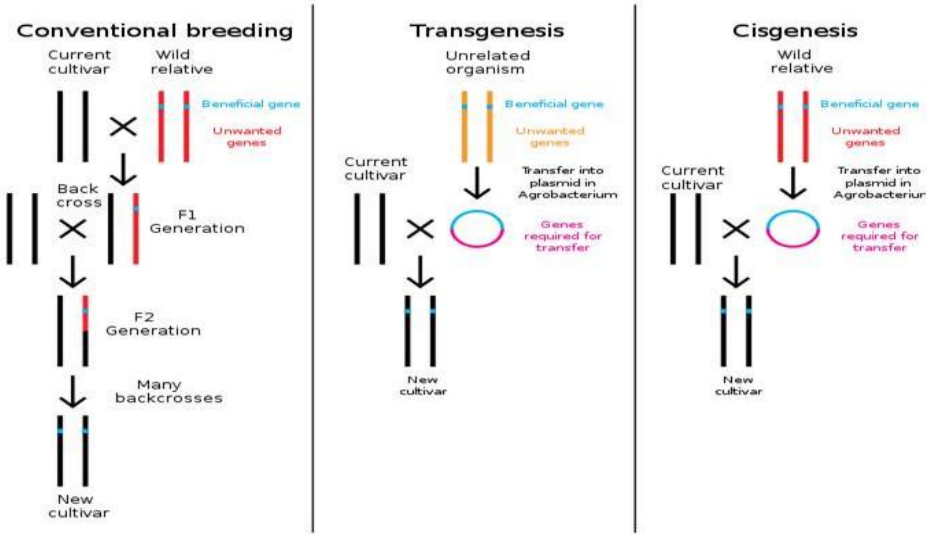

Figure 8. Comparative illustration of conventional breeding, transgenesis and cisgenesis (Lusser et al., 2011)

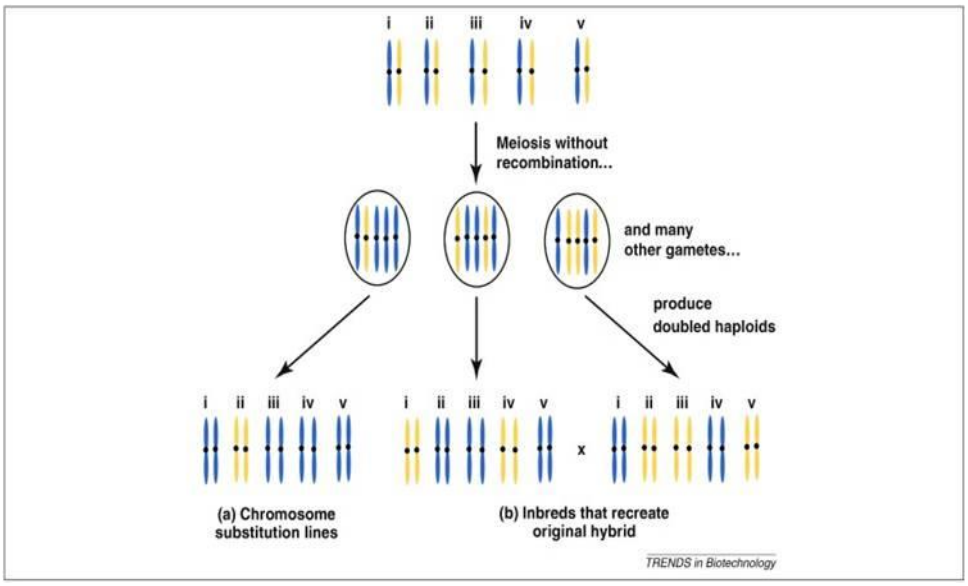

Figure 9. The conceptual representation of the reverse breeding technique (Lusser et al., 2011)

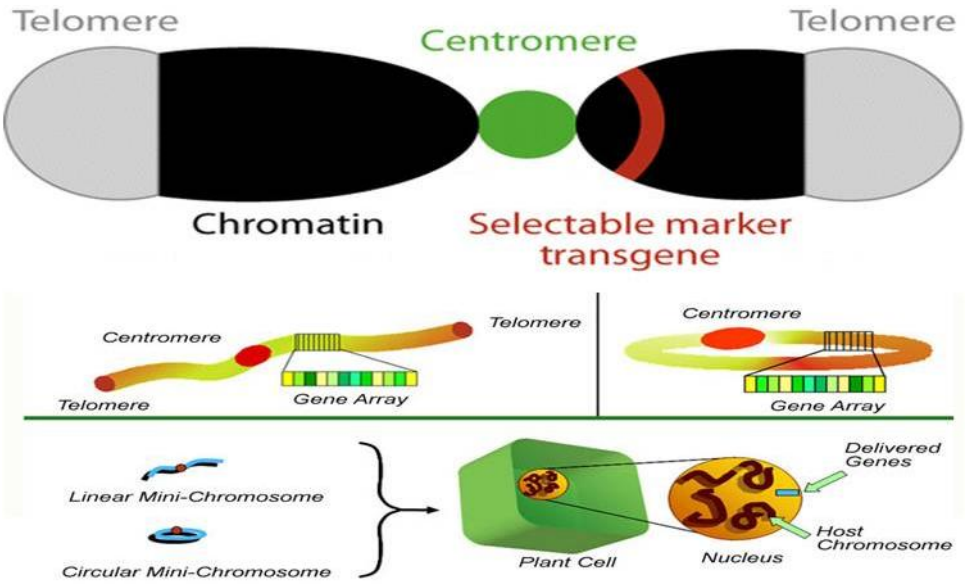

Figure 10. Assembly of synthetic mini-chromosome with desired gene-cassettes (Gaeta et al., 2011; Al Fin Energy Blog)

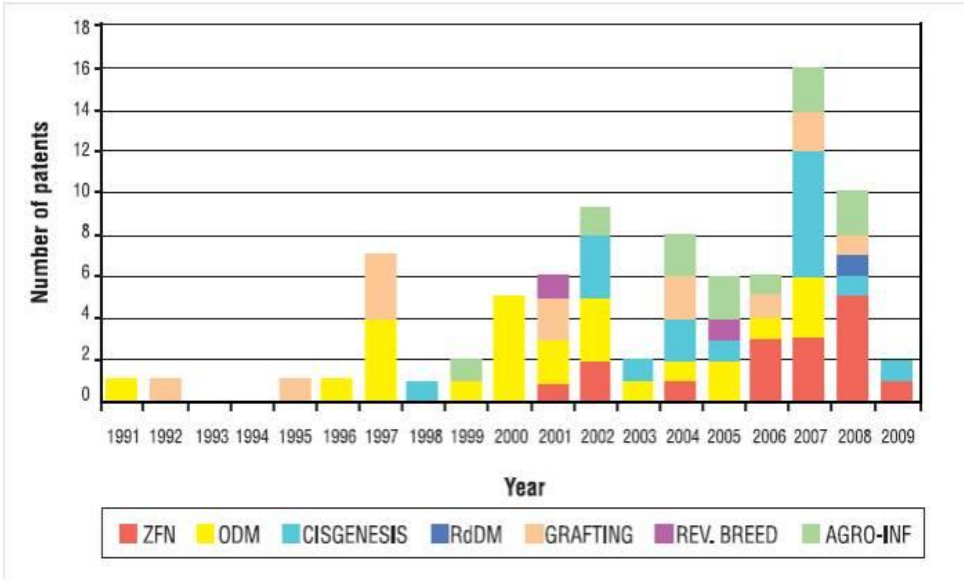

Figure 11. Number of patents related to the new generation plant breeding techniques during the past decade (Lusser et al., 2011)

skilful art to more and more molecular biology dependent; but the question remains - how many of these are breeder friendly to adopt in real time plant breeding? Do these tools of new biology make plant breeder shift his solo art based on strong phenotype association with the plants to molecular breeding that warrants competent team work?

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