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Review article

IMPROVEMENT OF FIELD CROPS THROUGH BIOTECHNOLOGY METHODS

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ABSTRACT

The degree of diversity present in a sample of germplasm can be measured in terms of morphology, pedigree, allelic diversity at marker loci, and allelic at genes determining target phenotypes. The environment often influences morphological characters and there may be limited polymorphism in cultivated germplasm. Pedigree information is not available for wild or crop progenitor germplasm, and even within cultivated germplasm, it can be difficult to differentiate between closely related accessions because complete pedigree records are not always available. Allelic diversity based on genes determining key phenotypes currently is not feasible in most crop plants, although the rapidly expanding EST and SNP databases will eventually make this feasible in some germplasm, diversity at marker loci is currently the most feasible strategy for characterizing diversity in wild and cultivated germplasm. Many types of molecular markers have been used to characterize germplasm, with each method differing in principle, application, type and amount of polymorphism detected, and cost and time requirement. These include random amplification of polymorphic DNA (RAPDs), restriction fragment length polymorphism (RFLPs), amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs).

INTRODUCTION

Recent advances in biotechnology, particularly in cell and tissue culture with efficient procedure for the successful culture of somatic cells, pollen, protoplast and regeneration) and molecular biology (availability of series of marker genes, cloned DNA fragments, Direct DNA transfer methods, RFLP, RAPD, AFLP, SSR and SNP maps) have made genetic manipulations more precise and rapid.

These advances have opened up new vistas improving the productivity and stability of crop plants. These advance techniques of molecular biology offers the opportunities to accomplish breeding objectives in decent and more accurate way. The material includes the introduction of foreign genes from such diverse biological system as unrelated plants, animals and microorganisms. The availability of nucleic acid probes has permitted more precise and rapid screening of germplasm, monitoring of alien gene introgression and detection of pathotypes in the infected materials.

It has now become possible to tag genes for quantitative traits with molecular markers (RFLP, RAPD, AFLP, SSR and SNPs), which facilitate in selection of materials in even early generations. The markers are free from environmental effects and provide a best selection such marker.

The following biotechnological techniques can contribute to improve crop plants.

- anther culture for developing homozygous lines and reducing the time required for breeding a variety;

- embryo rescue for producing species hybrids and for overcoming incompatibility barriers;
- protoplast fusion by passing sexual hybridization and produc- tion of somatic hybrids and organelle recombinants;
- somaclonal variation and selection of useful mutants at cellular level;
- somatic embryogenesis for efficient genetic transformation and production of synthetic seeds;
- transfer of useful foreign genes and production of transgenic varieties;
- gene tagging with molecular markers to facilitate marker-based selection for complex agronomic traits;
- nucleic acid probes for rapid and precise diagnosis of plant pathogens and monitoring of alien gene introgression.

Biological Techniques in crop improvement:

Anther culture for developing homozygous lines: Anther culture refers to the *in vitro* culture of anthers containing microspore or immature pollen grains on a nutrient medium for the purpose of generating haploid plantlets. The chromosome number of haploid plantlet is doubled by an appropriate procedure to produce a completely homozygous diploid plant, referred as *doubled haploids* (Poehlman 1987). The conventional development of improved wheat and rice varieties involves growing large F_2 populations and selecting desirable lines in F_2 to F_7 segregating generations to ultimately develop homozygous lines. Haploid plants can be obtained through anther culture of F_1 hybrids, which become homozygous plants upon chromosome doubling, either spontaneously or through colchicines treatment. As a result of this, resources are saved the time required to develop new varieties from six to two years for complete homozygosity.

Methods are available for production of doubled haploid lines in wheat like anther culture (Chaung et al., 1978; de Buyser and Henry, 1979; Henry and de Buyser, 1980), or isolated microspore culture (Venkatanagappa 1993; Ding et al. 1991, 1995; Luckett et al. 1991; Darvey 1998; Kasha et al. 1998) and crossing with *Hordeum bulbosum* (Kasha and Kao, 1970; Barclay, 1975, Inagaki 1987), wheat crossed with maize system (Morshedi and Darvey, 1995; Morshedi et al., 1996, 1997; Darvey, 1998). In the former, anthers from post-meoitic spikes are incubated on a potato extract medium. Embryoids appear in 20-40 days and are transferred first on an appropriate growth medium and subsequently to potting compost. When they reach three tiller stage, they are treated with colchicines solution for chromosome doubling. In the *Hordeum bulbosum* technique, ears of wheat are fertilized with pollen from *Hordeum bulbosum,* the chromosomes of which are subsequently eliminated during development, leaving a haploid wheat embryo. This is excised and transferred to an agar medium 2-3 weeks after pollination. The plantlets are later transferred to potting soil and treated with colchicine in the anther culture technique.

The anther culture technique has been widely used for the last ten years, and has recently contributed to release of new wheat varieties in china

and France (Hu, et al. 1983; Hu, 1985; de Buyser et al.1987).

Embryo rescue for the production of species/generic hybrids: Wild hybridization (i.e. intergeneric hybridization) is the first step to introduce the alien variation and to transfer the desirable traits from wild species into cultivated species. The use of hybridization between *Triticum aestivum* L. or *T. turgidum* var. *durum and Dasypyrum villosum* Canddargy (syn. *Haynalidia vilosa)* to obtain new amphiploids or derivative wheat lines was started in 1903 by Strampelli (Strampelli 1932).

Wild species of rice are rich source of useful genes, whereas wild relatives have rarely been used in rice improvement, however, mainly because F_1 embroys are aborted when crosses between cultivated *Oryza sativa* (AA genome) and species with BB, CC, BBCC, CCDD, EE and FF genomes are accomplished. Nevertheless, several interspecific hybrids have been produced through the embryo rescue technique. When the embryos are ten days old, they are removed and incubated on a culture medium. After few days they start to germinate and produce healthy hybrid plants.

Protoplast fusion for production of somatic hybrids: Somatic cell hybridization, somatic cell fusion or protoplast fusion, refers to fusion of plant protoplasts (cells without cell wall) from somatic cells of different species and the regeneration of hybrid plants from the fused protoplasts. The procedure has been developed to generate the hybrids by somatic cells where seeds cannot be produced by sexual hybridization following wide crosses. It is now possible to regenerate plants from protoplasts of both japonica and indica rices (Yamada, et al., 1985; Abdullah, et al., 1986; Kyozuka, et al., 1987; Lee et al., 1989). Hayashi, et al., (1988) obtained somatic hybrids between *O. sativa and O. officinalis, O. elchingeri, O. brachyantha, O. perrieri* through protoplast fusion. This technique has not been fully exploited in wheat, the reasons are not known.

Somaclonal variation and selection of useful mutants at cellular level: Somaclonal variation refers to variation in plants derived from cell cultures and their progenies. Somaclonal variation has been demonstrated in a large number of crop species, such as wheat, rice, sugarcane, potato and tobacco (Larkin and Scowcroft, 1981). Somalonal variation is known to occur for a wide range of characteristics such as disease resistance, salinity tolerance, aluminum toxicity tolerance, plant height and growth duration.

Somatic embryogenesis: The establishment of embryogenic cell cultures is becoming increasingly important for obtaining frequency of plant regeneration from cultured cells, pollen grains and protoplasts, as well as transformed cells. Indeed somatic embyogenesis has proved to be a major breakthrough for achieving plant regeneration from protoplasts. Although somatic embryogenesis has been reported in more than 150 plant species, the frequency of plant regeneration in several of them is too low to be in practical use. Synthetic seed technology in rice has vast potential for exploiting hybrid vigour, as it would permit the multiplication and large-scale propagation of superior hybrids.

Transfer of useful foreign genes and production of transgenic plants: Recent advances in tissue culture and

molecular biology have opened up new vistas for introducing foreign genes into crop plants. These techniques may not only facilitate the transfer of cloned, well-defined single genes or parts of genes (Cocking et al., 1981; Schell et al. 1982), but also allow gene transfer from completely unrelated species. They may also enable the modification or replacement of undesirable genes (Shillito et al 1986), and thus introduce new economic potential to cultivated plants. Foreign genes have been transferred /incorporated through *Agrobacterium* as well as through direct DNA transfers methods (Uchimiya, et al., 1989).

Transgenic plants have been produced in about 30 species, including tobacco, petunia, *Arabidopsis*, tomato, potato, *Brassica*, soybean, phaseolus, cotton, lotus, lettuce, flax, celery, eggplant, populas, rice and maize. However, in most of these cases, marker genes of bacterial origin have been incorporated. In few cases, some agronomically useful genes such as that herbicide tolerance and for resistance to insects and viruses have been incorporated into the crop plants. The introduction of Bt (*Bacillus thuringiensis*) gene into cotton crop against insect resistance has been developed. Jones et al (2003) have reported the development of transgenic plants in wheat with variable T-DNA delivery and regeneration. Miroshnichenko, et al (2003) has developed transgenic lines of Russian wheat cultivars through particle-bombardment mediated transformation. Immature embryos derived explants were transformed with a plasmid *bar* and *gfp* genes either *alone* or in combination with another plasmid containing *gus* genes. The development and utilization of transgenic wheat plants for agronomically important characters were reported by a number of workers (Murai et al 2003; Pauk et al 2003; and Wu et al 2003).

Molecular Markers: Genomics can be defined as mapping, sequencing, and functional analysis of genomes, the entire genetic complement of an organism. Before high throughput DNA sequencing was available, the structural aspects of genomes were studied largely by mapping using genetic markers. Rice has been at the forefront of plant genomics because of its small genome size and relatively low amount of repetitive DNA, its diploid nature, and its ease in manipulation in tissue culture. Some of the milestones include (i) the development of the first restriction fragment length polymorphism (RFLP) map (McCouch et al 1988); (ii) the application of markers based on the polymerase chain reaction (PCR) such as random amplified polymorphic DNA (RAPD) (Zheng et al 1991), amplified fragment length polymorphism (AFLP) (Cho et al 1996; Mackill et al 1996), simple sequence repeat (SSR) markers (Wu and Tanksley 1993; Zhao and Kochert 1992, 1993); (iii) the identification of quantitative trait loci (QTLs) (Ahn et al 1993, Wang et al 1994); and (iv) map-based cloning of a disease resistance gene (Song et al 1995).

These molecular markers differ from the conventional or morphological markers in five inherent properties that increase their utility in plant breeding and the properties are:

- Genotypes of molecular loci can be determined at whole plant, tissues and even cellular levels. Pheno- types of most morphological markers can

only be differentiated at whole plant level.

- A relatively large number of naturally occurring alleles are found at molecular loci. Distin-guishable alleles at morphological markers loci occur less frequently and often must be induced through mutagenesis.
- There is no deleterious effect associated with alternate alleles of molecular markers. This is not in case with morphological markers, which are often accompanied by undesirable phenotypic effects.
- The alleles of most molecular markers are co-dominant; all the possible genotypes can be differ-entiated in any segregating genera-tions. Alleles at morphological marker loci usually interact in dominant recessive manner, prohibiting their use in many crosses.
- In morphological marker loci, strong epistatic effects limit the number of segregating markers that can be unequivocally scored in the same segregating generations. Very few epistatic or pleotropic effects are observed with molecular markers, thus a virtually limit less number of segregating markers can be monitored in a single population.
- The morphological markers are environment based; it can be changed with alternate environ-ments.However, molecular markers are free from such type of environment changes, which can easily be identified in a molecular assay.

Marker assisted selection (MAS): A significant advance in the practical utilization of molecular markers was the development of SSR (Simple sequence repeat) markers, also referred to as microsatellite markers (McCouch et al 1997), such as $(GT)_n$ or $(CT)_n$ are ubiquitously interspersed in euro-karyotic genomes (Tautz and Renz 1984). These markers are based on repeats of short DNA sequences (2-5 bp) that are highly polymorphic and easy to detect. This kind of polymorphism at specific loci is easily detectable using specific primers in the flaking regions of such loci and subsequent amplifications via the polymerase chain reaction (Weber and May 1989; Litt and Luty 1989). The high level of polymorphism, combined with a high interspersion rate, makes them an abundant source of genetic markers and extensive genetic maps have been constructed on this basis for human (Weissenbach et al 1992; Rohrer et al 1994; Bishop et al 1994; Barendse et al 1994). The advantages of microsatellite markers over other PCR-based marker systems result from their potential for automated analysis and their co-dominant nature (Rafalski and Tingey 1993; Jamali et al 2004).

In plants, the frequency and number of several microsatellite types have been estimated for tropical forest trees (Condit and Hubbell 1991), *Brassica* (Lagercrantz et al 1993), soybean (*Glycine max*), wheat (Adhikari et al 2004; Guiha et al 2004; Martin et al 2004; Cook et al 2004) and 34 other plant species (Morgante and Olivieri 1993). The genetic diversity studies were conducted in CIMMYT for maize inbred lines by using SSR markers (Xia et al 2005**).** The results from these studies indicate that $(AT)_n$ is far more abundant in plants than in mammalian species, where $(GT)_n$ is the most abundant microsatellite type. Studies involving PCR amplification of specific

loci and analysis of variability were performed for soybean (Akkaya et al 1992), rice (Wu and Tanksley 1993), maize (Senior and Heun 1993) and *Arabidopsis* (Bell and Ecker 1994). They indicated that microsatellites in plants can be up to tenfold more variable than other marker systems such as restriction fragment length polymorphisms (RFLPs). Microsatellites, may represent a very useful genetic marker system for the genetic mapping of species with little intraspecific polymorphism, such as most in-breeding species.

Mapping of agronomically important genes itself can provide useful information for plant breeders. The most common example is the disease resistance genes. While meticulous genetic studies identified the major genes controlling major-gene resistance to Japanese rice blast isolates (Kiyosawa 1974). The systematic study of resistance for rice tropical blast races was hindered by the existence of multiple genes in many varieties and isolate differences between countries.

Application of functional genomics: The sequencing of crop plants such, as rice and wheat genomes will facilitate the identification of many important genes. The forward genetics approach for identifying functionally important genes drives from a known allelic difference conferring improved phenotype. In such an approach, the objective is to identify a sequence change can then become the basis for a marker that is specific for that allele. These types of markers will always co-segregate with the trait of interest and should also be polymorphic in any cross. Such markers will often be based on a single nucleotide polymorphism (SNP). Numerous assays are available to detect these SNPs (Kirk et al 2002).

Identification of SNPs: SNPs can be discovered *in silico* when gene sequences are available from more than one allele of a particular gene. The rice genome sequence may also provide a valuable source of primer sequences for SNP discovery. Due to high similarity of gene order and gene sequences in rice and wheat, it is possible to use rice sequences to design PCR primers and amplify the corresponding genes in wheat. The rice genome sequence can be used to help target gaps in genetic maps or to isolate markers for multiple genes in particular regions of the genome. In this case, the primers should be designed to coding regions flanking introns where possible. This will increase the chance that primers will work across species and increase the chance of discriminating between amplification products from the different homeologous loci in wheat, which increase the chance of identifying SNPs.

SNP assays: There are many technologies available to detect or screen for known SNPs in populations, including, (1) Taqman (Morin et al 1999), (2) molecular beacons (Marras et al 1999), (3) fluorescent ddNTPs, (4) restriction enzyme polymorphism (Neff et al 1998), (5) MALDI-TOF mass spectroscopy (Griffin and Smith 2000), (6) microarry hybridizations (Hirschhorn et al 2000), (7) DHPLC (Gross et al 1999), and SNAP (Drenkard et al 2000). Which technique is used in a particular laboratory and this depends upon the scale of SNP analysis being undertaken. The use of florescent dideoxynucleotides or MALDI-TOF is likely to be the most useful for genetic

map construction, microarrys for intensive screening of germplasm, and molecular beacons and restriction enzyme polymorphisms for targeting small numbers of specific markers in implementation labora-tories.

CONCLUSION

The large scale application of biotechnological methods to plant breeding will result the new vistas that reduce the cost and increase the throughput of the assays. Gel or capillary-based DNA sequencers can be used in genotyping, but micro arrays or other non-gel systems may allow whole-genome analysis of large number of plants commonly grown in breeding programmes. Ultimately the objective will be to rapidly assay the genetic makeup of individual plants in breeding populations. Producing graphical genotypes (Young and Tanksley 1989) of each plant or progeny row will allow the breeder to determine which chromosome sections are inherited from each parent and will greatly expedite the process and minimize the need for extensive field tests. These new tools will greatly enhance, but not replace, the conventional breeding process.

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