

Wheat biotechnology: A minireview

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Due to the inherent difficulties associated with gene delivery into regenerable explants and recovery of plantlets with the introduced transgene, wheat was the last among cereals to be genetically transformed. This review attempts to summarize different efforts in the direction of achieving genetic transformation of wheat by various methods. Particle bombardment is the most widely employed procedure for the introduction of marker genes and also for the generation of transformed wheat with introduction of agronomically important genes for quality improvement, engineering of nuclear male sterility, transposon tagging, resistance to drought stress, resistance against fungal pathogens and insect resistance. The other methods of choice of gene delivery into wheat tissues include electroporation and co-cultivation with *Agrobacterium*. Several alternative approaches including microinjection, direct imbibition, permeabilization, silicon carbide fiber-mediated and pollen tube pathway have also been attempted for introduction of foreign DNA with varied degrees of success. In future, use of marker assisted selection and genomics approaches will increase the effectiveness and efficiency of wheat breeding programs, and will also provide insights into genetic control of key traits to be used for genetic manipulation.

abundant sources of energy and proteins for the world population. Ninety-five percent of wheat grown today is of the hexaploid type, used for the preparation of bread and other baked products. Nearly all of the remaining 5% is durum (tetraploid) wheat, which is mainly used for making pasta, macaroni and biscuits. Wheat is characterised by a large genome size (approximately 17000 Mb), thus making the improvement process by any method genetically challenging.

Modern bread wheat is a true breeding hybrid with its ancestry linked to three wild grass species still growing in the Middle East. Bread wheat first originated in the 'Fertile Crescent', an area in the Middle EAST, which stretches from Israel and Lebanon into Syria, Turkey, Iraq and Iran. Diploid einkorn types of wheat are the earliest and the most primitive, while the hexaploids including the bread wheat, *Triticum aestivum*, constitute the most recent and latest step in the evolution of the wheat complex. Millions of years ago, the first hybridisation event is thought to have occurred when the wild grass *Aegilops speltoides* crossed with the wild diploid wheat, *Triticum monococcum*. The resultant hybrid was the tetraploid emmer wheat, *Triticum dicoccum*. Domestication of emmer wheat led to the evolution of the durum wheat. Hybridisation of tetraploid durum wheat, *Triticum turgidum* var. *durum* (2n=28, AABB) with the diploid wild goat grass, *Aegilops tauschii*, led to the origin and evolution of hexaploid wheat about 8000 years ago. Bread wheat is thus an allohexaploid, containing

Wheat is a member of the family Poaceae which includes major cereal crops of the world such as maize, wheat, and rice. Among the food crops, wheat is one of the most

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three distinct but genetically related (homoeologous) copies each of the three originally independent haploid genomes, the A, B and D (Gill and Gill, 1994). With the evolution of wheat, civilizations thrived and spread from the Middle East to new continents, resulting in massive wheat production feeding the growing world population. The tribe Triticeae comprising of over 300 species including wheat, rye and barley is considered as germplasm source for wheat improvement by classical breeding approaches.

Scientific approaches to crop improvement have their history in the rediscovery of Mendel's law at the beginning of this century. Consistently since then, breeders have been searching for technologies and converting this 'art' of breeding to a 'science'. Wheat breeders have been able to introduce desirable traits that increased the grain yield and minimize the crop loss. However, conventional breeding techniques which are based on processes of crossing, back crossing and selection, proved to be time consuming and, therefore, could hardly keep pace with the rapid co-evolution of pathogenic micro-organisms and pests. The development of *in vitro* technologies have thus complemented the conventional methods of wheat breeding in generating genetic variability necessary for creating novel cultivars with desirable characters.

In recent years biotechnology is emerging as one of the latest tools of agricultural research. In concert with traditional plant breeding practices, biotechnology is contributing towards the development of novel methods to genetically alter and control plant development, plant performance and plant products. The term biotechnology is composed of two words bio (Greek *bios*, means life) and technology (Greek *technologia*, means systematic treatment). Biotechnology involves the systematic application of biological processes for the beneficial use. One of the areas of plant biotechnology involves the delivery, integration and expression of defined genes into plant cells, which can be grown in artificial culture media to regenerate plants. Thus biotechnological approaches have the potential to complement conventional methods of breeding by reducing the time taken to produce cultivars with improved characteristics. Conventional breeding utilizes domestic crop cultivars and related genera as a source of genes for improvement of existing cultivars, and this process involves the transfer of a set of genes from the donor to the recipient. In contrast, biotechnological approaches can transfer defined genes from any organism, thereby increase the gene pool available for improvement. The improvement of wheat by biotechnological approaches primarily involves introduction of exogenous genes in a heritable manner, and secondarily, the availability of genes that confer positive traits when genetically transferred into wheat.

The genetic improvement of wheat has received

considerable attention over the years from plant breeders with the purpose of increasing the grain yield and to minimize crop loss due to unfavourable environmental conditions, and attack by various pests and pathogens. In the early 60's, conventional breeding coupled with improved farm management practices led to a significant increase in world wheat production thereby ushering in the green revolution. Subsequently, the targets of genetic improvement shifted to reducing yield variability caused by various biotic and abiotic stresses and increasing the input-use efficiency (Pingali and Rajaram, 1999). With this change in the global food policy in the last few decades, biotechnology offered a possible solution firstly, by lowering the farm level production costs by making plants resistant to various abiotic and biotic stresses, and secondly, by enhancing the product quality (i.e. by increasing the appearance of end product, nutritional content or processing or storage characteristics). The introduction of foreign genes encoding for useful agronomic traits into commercial cultivars has resulted in saving precious time required for introgression of the desired trait from the wild relatives by conventional practices and alleviating the degradation of the environment due to the use of hazardous biocides. In recent years, wheat improvement efforts have therefore focussed on raising the yield potential, quality characteristics, resistance to biotic stresses and tolerance to abiotic stresses depending on the regional requirement of the crop.

Approaches for wheat transformation

The last two decades have witnessed the widespread use of varied approaches for introduction of exogenous DNA into wheat. Wheat improvement by genetic engineering requires the delivery, integration and expression of defined foreign genes into suitable regenerable explants. Initial attempts at introducing transgenes into wheat employed protoplasts as explants due to the absence of cell walls. The introduction of marker gene constructs into protoplasts provided valuable information regarding the expression pattern and tissue specificity of various promoters and regulatory elements in the transformed tissue. However, the difficulties associated with plantlet regeneration from protoplasts have compelled researchers to look for alternate target cells/tissues with better regeneration capabilities. Therefore, attention shifted to embryogenic suspension cells and embryogenic callus cultures derived from scutellar tissue of mature and immature embryos. A detailed and in depth account of the *in vitro* culture response of wheat from different explants is available in numerous reviews and so has not been dealt with in detail here (Bajaj, 1990; Maheshwari et al. 1995; Vasil and Vasil, 1999). In recent years, with the development of suitable regeneration

protocols, microspore embryos and immature inflorescences are emerging as suitable target tissues for genetic transformation experiments. Till date, the biolistics approach has been most successful in delivering foreign genes into wheat.

Scorable markers

Initial steps for genetic transformation involves delivery of a gene cassette into recipient cells followed by analysis of the expression of delivered gene. The results of the above events can be detected by assaying the expression of a reporter gene introduced into plant cell cultures or intact tissues. The reporter genes produce a visible effect, directly or indirectly, due to their activity in the transformed cells. Analysis of reporter gene expression does not require the integration of the transgene into the host genome and is commonly used to test promoter and gene functions. Initial studies on the introduction of foreign DNA to wheat have relied on the use of the *E. coli* gene for the enzyme chloramphenicol acetyl transferase (*cat*) (Hauptmann et al. 1988; Chibbar et al. 1991). However, the detection of transgene by enzymatic and immunochemical methods for measurement of CAT activity and amount of CAT protein, respectively, was tedious; and moreover, presence of inhibitors of CAT activity and endogenous CAT activity (Chibbar et al. 1991) hampered its use as a reporter gene in wheat. With the availability of a protocol for the rapid assay of β -glucuronidase (*gus*) gene from *E. coli*, this gene has emerged as the most widely used scorable marker in wheat transformation (Vasil et al. 1992; Vasil et al. 1993; Weeks et al. 1993; Becker et al. 1994). GUS enzyme hydrolyzes β glucuronide compounds and gives reaction products that can be quantified spectrophotometric or spectrofluorometrically (Jefferson et al. 1987). The *gus* reporter gene system is extremely useful for optimisation of parameters for genetic transformation, due to the availability of a simple histochemical detection procedure. One of the major limitations of *gus* reporter gene system, however, is the destructive nature of its assay. Thus, to study the fate of introduced transgenes in living cells, vital reporter genes encoding for anthocyanin biosynthesis, green fluorescent protein, and firefly luciferase have been used successfully. The R genes of *Zea mays* which stimulate endogenous anthocyanin accumulation in the vacuoles of plant tissues is useful as a scorable marker in mature and differentiated cells. Due to high sensitivity and ease of visualization, the use of R genes in wheat transformation have been reported by different groups (Kloti et al. 1993; Dhir et al. 1994; McCormac et al. 1998; Chawla et al. 1999a; Chawla et al. 1999b). A synthetic, spectrally modified, version of green fluorescent protein (*gfp*) from the jellyfish, *Aequorea victoria* has also been used as a vital marker in wheat

transformation (Pang et al. 1996; McCormac et al. 1998). Recently Jordan (2000) reported the use of a modified *gfp* as a visible marker for the detection of transgenic wheat plants on the basis of *gfp* expression alone. Amongst the other scorable markers, luciferase gene from the fire fly, *Photinus pyranus*, has been successfully used in stable transformation of wheat (Lonsdale et al. 1998; Harvey et al. 1999). Luciferase and modified versions of *gfp* permit non-destructive analysis of transgene activity, ease to follow the fate of introduced transgenes in individual tissue samples, facilitate rapid assessment and comparison of different transformation procedures, and provide valuable insight into the conditions influencing the efficiency of DNA integration and stable expression.

Selectable markers

The varied frequency of DNA delivery in cells of different explants has necessitated the development of methods for efficient selection of cells that carry and express the introduced gene sequences. The selection regimes for transformed cells are based on the expression of a gene termed as the selectable marker producing an enzyme that confers resistance to a cytotoxic substance often an antibiotic or a herbicide. The most commonly used selection marker in wheat transformation is the *bar* gene (bialaphos resistance gene) encoding for phosphinothricin acetyl transferase (*pat*). Both *bar* and *pat* genes isolated from different *Streptomyces* species, encode for phosphinothricin acetyl transferase. Amongst the antibiotic resistance markers, the bacterial neomycin phosphotransferase II (*nptII*) gene providing resistance to aminoglycoside antibiotics is commonly used in wheat transformation. Herbicide resistance genes offer an alternative to antibiotic-resistant markers. Genes coding for 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a critical enzyme for aromatic amino acid biosynthesis, and phosphinothricin acetyl transferase (PAT) provide tolerance to glyphosate and glufosinate ammonium herbicides, respectively. The enolpyruvylshikimate-phosphate synthase (CP4) gene isolated from *Agrobacterium* strain CP4 and the glyphosate oxidoreductase (GOX) also provide tolerance to glyphosate by degrading glyphosate into aminomethyl phosphoric acid, and have been added to the list of selectable markers available for wheat (Zhou et al. 1995). The hygromycin phosphotransferase (*hpt*) gene, widely used in rice transformation, has also been reported as an efficient selectable marker for achieving stable genetic transformation in wheat (Ortiz et al. 1996). The efficiency of *hpt* as a selectable marker is comparable to the other widely used selectable markers encoding resistance for antibiotics and herbicides (Table 1). Recently mannose-6-phosphate isomerase (MPI), encoded by *manA* from *E. coli*, has been

developed as a selectable marker for wheat. This marker allows transformants to grow on mannose as a sole carbon source and has a positive mode of action which encourages the growth of the transformed tissues rather than just permitting it (Hansen and Wright, 1999). Recently, Weeks (2000) reported the use of cyanamide hydratase (*Cah*) gene as a selectable marker for wheat transformation. The *Cah* gene gives the transformed tissues the ability to grow on cyanamide-containing media by converting cyanamide into urea (which can be used as a fertilizer source).

Transformation of wheat protoplasts

Protoplasts are regarded as competent targets for gene transfer due to the absence of cell walls. During the early 80's protoplasts were the chosen explants for wheat transformation and their use as explants for direct gene transfer was facilitated by the development of suitable techniques for isolation of protoplasts from different tissues. However, due to limited success with culturing protoplasts from organized tissues (Mahalakshmi et al. 1993), cell suspension/calli derived protoplasts have been used in wheat. To overcome the hindrance exercised by the plasma membrane, protoplasts are subjected to either chemical treatment [polyethylene glycol (PEG)] or subjected to physical forces like electric pulses (electroporation), either alone or in combination with PEG, for the introduction of foreign DNA. For wheat, isolation of protoplasts from embryogenic callus cultures have been the most popular tissues of choice.

The first report of direct gene transfer in wheat protoplasts was by Lorz et al. (1985) from cultured cells *Triticum monococcum* by PEG-mediated uptake. The transformed cells were selected on a kanamycin-containing medium and identified by detection of *nptII* activity. Subsequently, Werr and Lorz (1986) reported the expression of the introduced genes but the majority of foreign DNA introduced was found to remain extra-chromosomal and transient gene activity was found to be dependent on the presence of the promoter fragment of the *Shrunken* gene of maize. This observation also indicated for the first time, the efficacy of a cereal promoter. Lee et al. (1989) introduced chimeric gene constructs having the *cat* reporter gene under the control of CaMV 35S promoter in aleurone protoplasts isolated from developing caryopses. The resultant gene activity detected in protoplasts from developing aleurone layers raised the possibility of investigating tissue- and development-specific control of genes for cereal seed proteins. By and large, the physiological age of the developing grain and the physiological state of the isolated protoplasts affected the level of transient gene expression. The significant factors affecting the transient activity of the reporter gene

constructs were the presence of the divalent cation Mg^{2+} with PEG, time lapse after DNA uptake, pre-culture medium and the regulatory elements of the vector construct. Nonetheless, these reports yielded low transformation efficiencies in the order of 0.005% and the low regenerating ability of the explant hampered work in this area.

Introduction of the *E. coli* plasmid, pCGN1055, containing the *hpt* gene into wheat protoplasts, by cationic liposome-mediated transformation resulted in the production of transgenic albino plantlets. The presence and activity of the transgenes was confirmed by assay of enzymatic activity and Southern hybridisation (Zhu et al. 1993) and the transformation efficiencies reported were in the order of 6%.

Electroporation of protoplasts

Electroporation is a technique that utilizes a high intensity electric pulse to create transient pores in the cell membrane thereby facilitating the uptake of macromolecules like DNA. Ou-Lee et al. (1986) reported the expression of the bacterial chloramphenicol acetyl transferase (*cat*) gene in three important graminaceous plants, i.e. rice, sorghum and wheat. The survival percentage of protoplasts after electroporation depended largely on the tissue of origin. The transient activity of *cat* gene was equal when it was fused to either CaMV 35S promoter or the *copia* long terminal repeat promoter of *Drosophila*. This study also demonstrated, interestingly, that the *Drosophila* promoter (*copia*) was capable of directing the synthesis of functional CAT enzyme in plant cells as efficiently as one of the strongest constitutive promoters known to function in plant cells. Besides the promoter influencing the foreign gene expression levels, presence of an intron was also found to be helpful as shown by Oard et al. (1989) with a maize alcohol dehydrogenase (*Adh*) intron in chimeric constructs. This was evidenced by ribonuclease protection assays that demonstrated increased mRNA levels for the intron-containing constructs versus those without the intron. This report highlighted the use of regulatory elements for enhancing the transgene expression. Zaghmout and Trolinder (1993) optimised other conditions for a significant increase in *gus* activity which included the protoplast source, the promoter, the PEG concentration, as well as, electroporation parameters especially the electric field strength, preincubation with the plasmid, and recovery period on ice after the electric pulse.

Stable transformation by electroporation (Zhou et al. 1993) was reported by the use of the plasmid pBARGUS into protoplasts isolated from cell suspension initiated from an anther-derived callus. This was the first report of stable transformation of wheat protoplasts and also confirmed by

Southern hybridization. Stable transformation of protoplasts was also reported by He et al. (1994) by electroporation employing the *bar* gene as the selectable marker, which also facilitated the selection of phosphinothricin-resistant colonies. Southern analysis and PAT assay revealed the presence of the *bar* gene and its product, respectively, in the regenerated colonies and plants. He et al. (1994) obtained transient expression of *gus* gene at a frequency of 1×10^{-5} which was more as compared to that observed by Lee et al. (1989). Electroporation of protoplasts thus considerably improved the gene transfer efficiency as compared to that achieved with PEG mediated approach.

Electroporation of organized tissues

The technique of electroporation, initially utilized for the introduction of foreign genes into protoplasts, was later extended for the transformation of organized tissues. In wheat, Kloti et al. (1993) reported the transfer of reporter genes by electroporating zygotic wheat embryos. The embryos were arranged with the scutella side facing the negative electrode, so that the negatively charged plasmid DNA will contact the target cells while moving towards the positive electrode. Transient expression of the anthocyanin regulatory gene and the *gus* gene facilitated the identification of transformed scutellum cells. Introduction of *gus* gene was also reported by electroporating organized tissue from slow-growing, embryogenic calli (Zaghmout, 1994). A detailed analysis of the various parameters affecting DNA delivery was undertaken by He and Lazzeri, (1998). This study identified and optimised the conditions for tissue electroporation of scutellum cells. The factors which played a significant role in affecting the electroporation efficiency were the electroporation voltage, the pulse length, volume of the electroporation buffer, osmoticum of the electroporation buffer, and medium, the osmoticum of the pre-electroporation medium and the pre-electroporation incubation time and temperature. Recently, the production of fertile transgenic wheat plants by this method from intact immature embryos was reported by Sorokin et al. (2000) at a transformation frequency of 0.4%. Molecular evidence confirmed the stable integration and the inheritance of the *gus* and *bar* transgenes in the T_0 and T_1 plants. The electroporation efficiency was found to be dependent on a combination of factors including pretreatments of the recipient tissue and the culture conditions. It thus appears that tissue electroporation may emerge as a routine transformation method for wheat due to its technical simplicity and low cost associated with this procedure.

Particle bombardment

The development of methodologies for the delivery of genes into intact plant tissues by particle bombardment has,

in fact, revolutionized the field of plant transformation. The concept of accelerating DNA-coated particles into cells and tissues has evolved from novelty to an established tool in plant molecular biology (Klein et al. 1987; Klein and Jones, 1999). This method of introducing DNA into cells by physical means was developed to overcome the biological limitations of *Agrobacterium* and the difficulties associated with plant regeneration from protoplasts. Initial studies on particle bombardment as a gene delivery method achieved transient expression of *gus* gene following bombardment of cell suspensions (Wang et al. 1988), leaf bases and apical tissues (Oard et al. 1990), immature embryos (Chibbar et al. 1991). Vasil et al. (1991) obtained stably transformed callus lines that expressed all the marker genes tested (*gus*, *nptII* and *EPSPS*). First successful generation of transgenic wheat plants was reported by Vasil et al. (1992) by particle bombardment of plasmid vector pBARGUS into cells of type-C, long-term regenerable embryogenic callus. Time required from isolation of immature embryos to the production of transgenic plants was 12-15 months. Subsequent improvement in procedures reduced the time required for production of transgenic wheat plants to 5-7 months by bombardment of immature embryos and embryogenic calli (Vasil et al. 1993), about 4 months by immature embryos (Weeks et al. 1993), 3 months by using an enhanced regeneration system using isolated scutella as the starting explant (Nehra et al. 1994), and more recently to 56-66 days by bombardment of cultured immature embryos (Altpeter et al. 1996a). Most of the subsequent gene delivery studies employed immature embryos, isolated scutellum, and calli initiated from immature embryos as the target tissue for bombardment (Table 2). The first successful transformation of durum wheat was reported by Bommineni et al. (1997) by particle bombardment of isolated scutella. Particle bombardment of explants like pollen embryos (Shimada et al. 1991), microspore-derived embryos (Loeb and Reynolds, 1994; Ingram et al. 1999) resulted in successful introduction of transgenes as evidenced by transient expression of *gus* gene, however regeneration of plantlets from these explants has not been reported. Iglesias et al. (1994) reported the direct delivery of foreign DNA to the meristem cells of immature embryos by microtargeting and transgenic sectors were obtained in both coleoptiles and leaf primordial. Notable here is the microtargeting of genes into meristematic cells which, nonetheless, were 10-fold lower in expression than the surrounding tissues. The effects of various bombardment parameters like amount of plasmid DNA, spermidine concentration, acceleration and vacuum pressure, osmotic pretreatment of target tissues on gene delivery into wheat tissues have been investigated in detail (Rasco-Gaunt et al. 1999) and most of the studies have found no clear correlation between transient expression and stable transformation. The transgenes introduced by

biolistic approach display a considerable degree of stability in integration and expression in subsequent generations (Altpeter et al. 1996b; Srivastava et al. 1996).

Since multiple copy insertions of the transgene is common in transgenics obtained by particle bombardment, Srivastava et al. (1999) recently reported the use of a strategy based on site-specific recombination of Cre-lox system to achieve single copy insertion in transgenic wheat. Bombardment of immature embryos with a transformation vector that consists of a transgene flanked by recombination sites in inverted orientations, results in recombination between outermost sites thereby resolving the integrated molecules into a single copy in the transgenic plants.

In recent years, particle bombardment has emerged as a reproducible method for the introduction of various marker genes in wheat (Table 2) and this method is successfully being used for the generation of transformed wheat with introduction of agronomically important genes for quality improvement (Altpeter et al. 1996b; Blechl and Anderson, 1996), engineering of nuclear male sterility (De Block et al. 1997), transposon tagging (Takumi et al. 1999), resistance to drought stress (Sivamani et al. 2000), resistance against fungal pathogens (Leckband and Lorz, 1998; Bliffeld et al. 1999; Chen et al. 1999; Bieri et al. 2000) and insect resistance (Altpeter et al. 1999; Stoger et al. 1999).

***Agrobacterium*-mediated transformation**

Agrobacterium -mediated transformation is a simple, low cost and highly efficient alternative to direct gene delivery methods. The advantages of the *Agrobacterium* system include the defined insertion of a discrete segment of DNA into the recipient genome. The initial studies on *Agrobacterium* and wheat were aided by the phenomenon of agroinfection, which for the first time demonstrated that the soil bacterium can interact in a limited manner with the cells of a monocot plant (Woolston et al. 1988; Dale et al. 1989) and transfer DNA. Agroinfection is the introduction of viral DNA residing between the T-DNA border sequences of *Agrobacterium* into plant cells and provide a suitable system to monitor DNA delivery into target explants by detection of disease symptoms. The wheat dwarf virus (WDV) acted as a sensitive marker for studying the wheat-*Agrobacterium* interaction due to amplification of viral DNA leading to the production of symptoms and detection by ELISA. The successful agroinfection of WDV paved way for a natural progression of techniques for the introduction of non-viral constructs with scorable and selectable markers, and also for a better understanding of parameters required for the introduction of foreign DNA

into wheat.

Most of the experiments on *Agrobacterium*-mediated transformation employed incubation of suitable explants with the *Agrobacterium* inoculum (Table 3). Mooney et al. (1991) for the first time demonstrated that wounding is not necessary for adherence of bacteria to explants of wheat and reported an increase in adherence of bacteria at the wound site caused by mechanical and enzymatic treatments. Chen and Dale (1992) reported a higher frequency of infection in exposed apical meristems of dry wheat seeds as compared to intact seeds. Mahalakshmi and Khurana (1995) tested the suitability of various explants for *Agrobacterium*-mediated gene delivery and reported an increase in transient expression of GUS gene in mature seeds subjected to mechanical wounding by abrasion as compared to intact seeds. The induction of *vir* genes which is necessary for gene transfer has been achieved by wounding and also by using chemical inducers like acetosyringone. Other factors influencing this association are also discussed by Mahalakshmi and Khurana, (1997). Significant progress made in the area of *Agrobacterium*-mediated transformation of rice and maize (Chan et al. 1993; Hiei et al. 1994; Ishida et al. 1996) has contributed tremendously to an increasing understanding of various parameters necessary for the successful generation of transgenic cereals. Cheng et al. (1997) first reported the stable transformation of wheat by *Agrobacterium*-mediated co-cultivation and demonstrated the successful transmission of the transgene to the next generation. This study developed a system for the production of transgenic plants within a total time of 2.5 to 3 months by co-cultivating freshly isolated immature embryos, precultured immature embryos and embryogenic calli. The factors which influenced the generation of transgenic plants included the explant tissue, inducers in the inoculation and co-cultivation media, and the surfactants present in the inoculation medium. Approximately 35% of the transgenic plants received a single copy of the transgene and one to five copies of the transgene were integrated into the wheat genome without rearrangement (Cheng et al. 1997). McCormac et al. (1998) used cell autonomous reporter genes (anthocyanin biosynthesis regulatory genes, *gus*, synthetic *gfp* for studying the localized transformation events. The localization of the transformed cells revealed a non-random distribution throughout each embryo and callus piece. Recently, *Agrobacterium*-mediated transformation of immature inflorescence tissue was reported by Amoah et al. (2001). This study achieved optimal T-DNA delivery (as measured by *gus* activity) in explants precultured for 21 days and sonicated (subjected to brief periods of ultrasound in presence of *Agrobacterium*). In near future, it can be expected that *Agrobacterium* will be employed as a reliable, efficient and economical vector for the introduction

of exogenous genes into wheat by various laboratories throughout the world.

Alternative approaches

Most of the alternative approaches for wheat transformation have attempted to develop a genotype independent, cost effective procedure for the introduction of foreign DNA into wheat. Prominent among these methods are microinjection, direct imbibition, permeabilization, silicon carbide fiber-mediated and pollen tube pathway; which have been used for wheat transformation with varied levels of success.

The pollen-tube pathway method for transferring foreign DNA into recently pollinated floret was first reported for the successful transformation of rice (Duan and Chen, 1985; Luo and Wu, 1989). In this approach the stigma is cut off and a drop of DNA solution is applied to the cut end of the style. The DNA reaches the ovule by flowing down the pollen-tube. The pollen-tube pathway was used to introduce a *verc203:gus* fusion expression cassette into the winter wheat plants, and Chong and coworkers (Chong et al. 1998) investigated the function of a vernalization-related gene *ver203*, by expression of a complementary DNA as an antisense RNA, in transgenic plants. Molecular evidence indicated the successful integration of the transgenes and elucidated the role of the VER203 protein in controlling heading and flower development in winter wheat (Chong et al. 1998).

The uptake of plasmid DNA through membranes of dry plant tissue, the physiochemical characteristics of which change during natural desiccation, was thought to be an alternative way of gene transfer. Uptake of plasmid DNA, as well as, recombinant wheat dwarf virus genome by imbibition as reported in seed derived dry embryos of wheat (Töpfer et al. 1989).

Simmonds and coworkers reported the use of microcapillaries for the introduction of plasmid DNA into the germ line precursor cells of apical meristems by microinjection, (Simmonds et al. 1992). Despite a positive indication by PCR amplification of DNA isolated from injected apices, this approach has failed to yield any transgenic plants.

The successful use of silicon carbide fibers in stable transformation of maize (Frame et al. 1994) raised the possibility of its use for the introduction of foreign DNA into wheat. In wheat, the preferential expression of the *gus* gene in the scutellum and the epiblast of mature embryos was observed following vortexing of the explants in a solution containing silicon carbide fibers and plasmid DNA

(Serik et al. 1996).

Recently, Mahalakshmi et al. (2000) exploited the altered membrane characteristics of dry tissues and coupled it with the use of membrane permeabilizing substances like saponin, toluene, triton, etc.; and reported *gus* gene expression in mechanically isolated seed-derived dry embryos of wheat by incubating in a buffer containing plasmid DNA along with various membrane permeabilizing agents. Although *gus* gene expression was reported as late as one month following permeabilization, recovery of plantlets was not reported. Nonetheless, all these methods promise the development of genotype independent methods of gene delivery and are thus of tremendous importance.

Introduction of useful traits by genetic transformation

Nutritional improvement

In addition to its basic calorific value, wheat with its high protein content is an important source of plant protein in the human diet. One of the prominent targets for the application of transgene technology for the nutritional improvement of wheat is targeted at enhancing the grain quality by, i) increasing the protein content, ii) increasing essential amino acids such as lysine, iii) increasing the high molecular weight (HMW) glutenins to improve breadmaking properties of wheat flour, iv) modifying starch composition and v) producing pharma- and nutraceuticals.

Amongst the cereals, the flour of bread wheat, *Triticum aestivum*, has a superior capability of forming leavened bread. This superiority stems from the structure and composition of its seed storage proteins, which upon hydration can interact to form gluten, an insoluble, but highly hydrated, visco-elastic aggregate that endows the wheat dough with its unique properties. Although the majority of wheat seed storage proteins participate in gluten formation, biochemical and genetic evidence has demonstrated that high molecular weight glutenin subunit (HMW-GS) plays a major role in determining the visco-elastic properties thereby determining bread making qualities. The HMW glutenins are necessary to create a strong dough, which is essential for making high quality, yeast-raised breads. Strong dough traps tiny bubbles of carbon dioxide gas formed naturally by yeast during mixing and subsequent raising thereby enabling the dough to rise forming leavened breads. Dough strength and the ability to contain gas bubbles is known as visco-elasticity and is an important characteristic of wheat with respect to its end product quality. Increasing understanding of the molecular basis of dough visco-elasticity would thus help in

developing strategies for minimizing the effect of unfavourable environmental factors on wheat end-use quality.

Genetic transformation of wheat is a key component in a scheme proposing a complete set of approaches to apply biotechnology to improve wheat quality via direct manipulation of HMW-glutenin genes (Vasil and Anderson, 1997). To alter the amount and composition of these proteins by genetic engineering, a gene encoding a novel, hybrid-subunit of HMW-GS under the control of native HMW-GS regulatory sequences was inserted into wheat using the biolistic approach (Blechl and Anderson, 1996). The HMW-GS *1Ax1* gene which is known to be associated with superior bread making quality, was introduced into the cultivar (Bobwhite) lacking this gene (Altpeter et al. 1996b). The introduced *1Ax1* gene under the control of HMW-GS promoter was expressed at high levels and stability was maintained for several generations. The results demonstrate the feasibility of manipulating the composition of wheat kernels by genetic engineering and have successfully made changes in both the levels and the types of seed storage proteins. This work also demonstrated the usefulness of tissue-specific promoters for the expression of transgenic proteins in the endosperm tissue of wheat. The prospect of achieving the elusive goal of nutritional improvement of wheat brightened further with the improvement in functional properties of wheat dough due to transformation of wheat with high molecular weight subunit genes. Transformation with one or two subunit genes results in stepwise increase in dough elasticity (Barro et al. 1997). The expression of a recombinant protein with x- and y- type HMW-GS subunit in transgenic wheat has resulted in altered gluten polymer assembly and composition. This study thus made it feasible to change the glutenin composition by expressing modified HMW-GS in transgenic plants (Shimoni et al. 1997).

The quality improvement studies were extended to *durum* wheat by He et al. 1999 with the introduction of one of the two subunits of HMW-GS subunit genes (*1Ax1* or *1Dx5*). Analysis of the expression of the additional subunits in the T₂ generation using a mixograph, indicated an increase of dough strength and stability. This study demonstrated the feasibility of manipulating durum wheat for superior bread and pasta making qualities (He et al. 1999). Similarly, the overexpression of the HMW subunit *1Dx5*, in transgenic wheat (*T. aestivum*) resulted in a four-fold increase in this proportion of component in the seed protein and also a corresponding increase in the proportions of the total HMW proteins and glutenins (Rooke et al. 1999). However, the overexpression of the *1Dx5* gene was found to be associated with a dramatic increase in dough strength by making it too strong, and, therefore, unsuitable for use in

conventional breadmaking. Recently, Alvarez et al. (2000) introduced the HMW-GS genes *1Ax1* and *1Dx5* into a commercial cultivar of *T. aestivum* that already expresses five subunits. The overexpression of *1Dx5* gene increases the contribution of the HMW-GS to a level of 22% of the total protein content. In near future, molecular approaches including genetic transformation and marker-assisted selection will provide an opportunity for improving further the wheat processing qualities.

Modification of wheat starch is presently targeted in various laboratories to improve its potential utility. Wheat grain is predominantly composed of starch, which is a mixture of two polymers, the almost linear amylose molecules, and the heavily branched amylopectin molecules. The ratio of amylose to amylopectin in starch determines its physico-chemical characteristics and thereby its end-use. Wheat flour, low in amylose content is desirable for noodlemaking as it improves noodle texture. In wheat, scientists are thus working towards increasing the amylopectin content of starch thereby reducing amylose content leading to the formation of a value added, low-amylose flour. The starch branching enzymes catalyse formation of 1, 6-linkages in the glucan polymer and control the amount of amylopectin produced. In recent years, efforts are underway towards detailed characterization of the starch branching enzymes (Baga et al. 1999b; Baga et al. 2000) and starch synthases (Li et al. 1999; Gao and Chibbar, 2000; Li et al. 2000). This increasing information and characterization of the various components of starch biosynthesis will enable researchers to make rational design of novel starches and alteration in starch levels in other crop plants as well (Slattery et al. 2000).

Wheat is widely used as an animal feed also for non-ruminants in several developed countries of the world. The phytase of *Aspergillus niger* is used as a supplement in animal feeds to improve the digestibility and also to improve the bioavailability of phosphate and minerals. The *phyA* gene from *Aspergillus niger*, encoding for the phytase enzyme has been successfully expressed in transgenic wheat lines by the microprojectile bombardment of immature embryos. The constitutively overexpressed phytase was found to accumulate at high levels in the endosperm (Brinch-Pederson et al. 2000). This work may thus open newer avenues for a wider applicability of wheat.

Wheat is also an ideal system for the production of novel compounds due to its excellent storage properties and the existence of an efficient processing industry. The potential products include the high value, low-volume compounds such as biologically active proteins and peptides, as well as, high volume, low-cost raw materials for packaging and

building (Shewry and Lazzeri, 1997). The production of recombinant antibodies in rice and wheat was recently reported with the expression of a medically important, single chain Fv recombinant antibody against the carcino-embryonic antigen. (Stoger et al. 2000). The recombinant antibody was targeted to the plant cell apoplast and endoplasmic reticulum and was detected in the leaves and seeds of wheat. More significantly, the recombinant antibodies remained active after prolonged seed storage at room temperature thus opening avenues for the further exploitation of wheat for producing high-value, novel compounds.

Engineering nuclear male sterility

The production of hybrids is an essential component of crop breeding programmes but till date, hybrid wheat has remained elusive! The development of a suitable hybridisation system for wheat requires a high degree of male sterility in all parts of the female parent to avoid self-fertilization. De Block and coworkers (De Block et al. 1997) have developed a nuclear male sterile system in wheat by introducing the *barnase* gene under the control of a tapetum specific promoter, the expression of which prevents normal pollen development at specific stages of anther development. This system employed the ribonuclease-inhibitor *barstar* gene to restore the fertility of male sterile plants. To avoid complicated gene integration patterns, the target tissues were incubated on niacinamide containing medium before bombardment. The authors suggest that the enzyme poly (ADP-ribose) polymerase (PARP), which plays a key role in the processes of cell division and recombination, is inhibited by niacinamide, thereby resulting in simple integration pattern of the transgene (De Block et al. 1997). Expressing the *barnase* gene at specific stages of anther development destroys the tapetum, thereby preventing normal pollen development and causes pollen sterility.

Resistance to biotic stress

The integration of transgenic approaches with classical breeding techniques offers a potential chemical-free and environment-friendly solution for controlling pests and pathogens. Wheat is attacked by a number of viral, bacterial and fungal pathogens and also by insect and nematode pests. Introgression of genes from the wild relatives exhibiting resistance to pests and pathogens has been successfully utilized over the years for generation of resistant varieties in wheat. With the development of plant transformation techniques, newer avenues for creating disease resistant and insect resistant crops have been created. Coupled with this is the availability of novel

transgenes encoding highly potential anti-microbial peptides, defense-related proteins and enzymes for the production of anti-microbial compounds in crop plants, have greatly enhancing the possibility of engineering crop plants for resistance to pests and pathogens (Shah, 1997; Sharma et al. 2000).

Fungal pathogens of wheat cause severe crop damages by infecting the spikes, leaves and roots. Amongst the various strategies to introduce fungal resistance by transgenic approach, strengthening the host plant defense by genetic manipulation hold tremendous potential. Biochemical and structural responses against fungal attack include reinforcement of plant cell wall, accumulation of phytoalexins with microbial toxicity, ribosome-inactivating proteins (RIP) that inhibit protein synthesis, antimicrobial peptides and synthesis of other PR proteins, (Yang et al. 1997). Genetic engineering allows the expression of foreign genes from distant unrelated species as well as the modification of the usual pattern of expression of an already present gene.

Most of the works on genetic engineering of wheat for resistance against biotic stress have focussed on developing protection against fungal pathogens (Table 4). Introduction genes encoding for chitinases from barley resulted in increased resistance against *Erysiphe graminis* (Bliffeld et al. 1999). Bliffeld et al. (1999) reported the adverse effect of a ribosome inactivating protein on plant regeneration and development. Nonetheless, moderate protection against *Erysiphe graminis* was reported with the transformation of wheat with a gene encoding for a ribosome inactivating protein from barley (Bieri et al. 2000). The genes encoding for thaumatin like protein (TLP) and stilbene synthase in transgenic wheat have been shown to improve resistance of T₁, T₂ progeny plants against the fungal pathogens (Leckband and Lorz, 1998; Chen et al. 1999). An increase in endogenous resistance against *Tilletia tritici* was achieved with the introduction of virally encoded antifungal protein (Clausen et al. 2000).

For the engineering of resistance against different pests and pathogens in wheat, genes encoding for viral coat proteins, antifungal proteins, and proteinase inhibitors have been successfully introduced (Table 4). Most of the introduced genes confer increased resistance to the corresponding pests and pathogens in the transgenic plants. Introduction of candidate genes (lectin, proteinase inhibitor) for insect resistance into wheat have resulted in growth inhibition of insects on transgenic seeds, thereby decreasing the fecundity of insect population.

Resistance to abiotic stress

Traditional approaches at transferring resistance to crop plants are limited by the complexity of stress tolerance traits, as most of these are quantitatively linked traits (QTLs). Nonetheless, the direct introduction of a small number of genes by genetic engineering offers convenient alternative and a rapid approach for the improvement of stress tolerance. Although, present engineering strategies rely on the transfer of one or several genes that encode either biochemical pathways or endpoints of signalling pathways, these gene products provide some protection, either directly, or indirectly, against environmental stresses.

Drought is a major abiotic factor that limits crop productivity, thereby causing enormous loss. The genes encoding the late embryogenesis proteins (LEA) which accumulate during seed desiccation, and in vegetative tissues when plants experience water deficiencies have recently emerged as attractive candidates for engineering of drought tolerance. Transgenic approach has been used for successfully introducing and overexpressing the barley *HVA1* gene encoding for a late embryogenesis abundant (LEA) protein by Sivamani et al. (2000) into wheat by particle bombardment. Most of the transgenic lines tested displayed improvement in important agronomic traits, including total dry mass and water use efficiency, shoot dry weight, root fresh and dry weights, when plants are grown under soil water deficit conditions. In general, this investigation showed that the transgenic lines expressing the *HVA1* gene had improved growth characteristics including an enhanced biomass yield under water deficit conditions. The discovery of novel genes, determination of their expression pattern in response to abiotic stress and an improved understanding of their roles in stress adaptation (obtained by the use of functional genomics) will provide the basis of effective engineering strategies leading to greater stress tolerance (Cushman and Bohnert, 2000).

Transgene silencing in wheat

Success at developing improved wheat cultivars through genetic engineering depends on stable and predictable expression of the inserted gene. However, gene silencing is a common phenomenon in the production of transgenic plants and needs to be effectively controlled for the desired results. This is all the more important because gene silencing is an important phenomenon involved during natural plant defense. Gene silencing is a complicated phenomenon as it includes both transcriptional gene inactivation and post transcriptional gene inactivation (Khurana et al. 1998). The complex and the large genome size of wheat is expected to be prone to silencing by introduced transgenes. With the development of transformation methodologies for wheat, more information

has started to accumulate regarding the inheritance and expression of the introduced transgenes. Information regarding the long term stability of transgenes is thus of immense significance for the use of genetic manipulation as a tool in wheat crop improvement and is necessary for the introduction and subsequent expression of desirable agronomic trait.

DNA methylation plays a significant role in establishing and maintaining an inactive state of the gene by rendering the chromatin structure inaccessible to the transcription machinery (Razin, 1988). Methylation of DNA is expected to result in reduced gene expression. Muller et al. (1996) studied the variability of transgene expression in clonal cell lines of wheat and found a negative correlation between the degree of methylation and marker gene expression. PEG-mediated approach was used to transform protoplasts isolated from suspension cultures of *T. aestivum*. The integration and expression of the selectable marker gene *nptII* fused with different promoters, namely, alcohol dehydrogenase, *shrunk* from maize, and *actin1* from rice, was confirmed by Southern analysis and enzyme activity test. Transgenic cell lines under selection were 'protoplasted' and clonal callus lines were cultivated from genetically identical single cells without selection pressure. A reduction/loss of marker gene expression was observed due to a reduction in the *nptII* transcript level and was seen to be associated with hypermethylation of the integrated DNA. The silencing effect was reversed by a 4-week culture phase on media supplemented with demethylation agent, 5-azacytidine, thus confirming the role of methylation in transgene silencing.

Demeke et al. (1999) studied the inheritance and stability of an *Act1D-uidA:nptII* expression cassette in T_4 and T_5 transgenic plants. Based on the histochemical localization of GUS activity, this study demonstrated the lack of any cytoplasmic effect on the inheritance of the transgene. The transgenes maintained a multiple integration pattern similar to that observed in the T_1 generation. The transgenic plants which produced low *gus* and *nptII* activity in seeds had an intact expression cassette. Southern blot analysis of genomic DNA performed with the methylation sensitive enzyme, *HpaII*, showed the transgene in GUS negative plants to be highly methylated relative to the transgene in GUS positive plants. Some of the studies on gene silencing also indicate that multiple integration pattern and copy number is also associated with DNA methylation (Hobbs et al. 1993; Muller et al. 1996).

Cannell and coworkers studied the inheritance of *gus* and *bar* marker genes over three generations. The integration, inheritance and expression of these marker genes in the

population studied were stable and predictable with a few exceptions (Cannell et al. 1999). The inheritance of integration patterns was stable, and transmission/inheritance of the transgenes followed Mendelian ratios in a majority of lines. From this study, the authors claim that the transformation procedure, transgene integration, and marker gene expression had little effect on the transmission of transgenes to the subsequent progenies. However, over the three generations studied, a uniform, 'progressive' transgene silencing, specific to the *gus* gene, was observed. This gradual loss of the *gus* gene expression was not accompanied by a reduction in *bar* expression. Since in this case, the *gus* and the *bar* genes are located on the same plasmid, observations indicate a post-transcriptional gene silencing.

The silencing of HMW glutenins was also observed in transgenic wheat expressing extra HMW subunits (Alvarez et al. 2000). Characterization of six independent events involving the transformation of wheat HMW-GS genes, 1Ax1 and 1Dx5, in a cultivar expressing five subunits by particle bombardment resulted in partial or complete gene silencing. Silencing of all the HMW glutenin subunits was observed in two different events of transgenic wheat expressing the 1Ax1 subunit transgene and overexpressing the 1Dx5 subunit. Control of gene silencing thus remains a challenge for the immediate future.

Meyer, (1995) advocated that the problem of gene silencing in wheat can be minimized by optimising methods for simple integration patterns, use of promoters and gene sequences isolated from cereals, use of matrix associated regions (MARs) or scaffold attachment regions (SARs), which insulate transgenes from surrounding chromatin, might help in reducing the gene silencing problem (Baga et al. 1999a).

Transposon tagging in wheat

Transposon mutagenesis has been widely exploited in various organisms to isolate genes that encode unidentified products (Sundaresan, 1996). The maize activator (*Ac*) and Dissociation (*Ds*) elements are the best-studied transposable elements in heterologous host plants. Initial studies have reported the introduction and activation of the *Ac/Ds* elements into cultured wheat cells by using a wheat dwarf virus by particle bombardment (Laufs et al. 1990; Takumi, 1996). Takumi and coworkers (Takumi et al. 1999) has developed a transposon tagging system in wheat by introducing the *Ac* transposase gene under the CaMV 35S promoter into cultured wheat embryos by particle bombardment. For the development of a transposon tagging system, embryos isolated from a stable *Ac* line were bombarded with a plasmid containing the maize dissociator (*Ds*) element located between the rice *Act1* and the *gus*

genes. The transient expression of the *gus* gene was observed after the excision of *Ds* elements. Southern and northern analysis of the T₀ and T₁ plants has exhibited the stable expression and inheritance of the *Ac* transposase gene. These results have demonstrated the precise processing of the maize *Ac* transposase gene and the synthesis of the transposase protein in transgenic *Ac* lines. The *Ac* transposase gene causes the transactivation and excision of the *Ds* in the *Ac* transgenic lines transformed with the maize *Ds* elements. Thus in near future we can expect traits of commercial importance to be tagged for a wider utility in important crop plants.

Marker assisted selection in wheat breeding

Conventionally, plant breeding depends upon morphological/phenotypic markers for the identification of agronomic traits. With the development of methodologies for the analysis of plant gene structure and function, molecular markers have been utilized for identification of traits. Molecular markers act as DNA signposts to locate the gene(s) for a trait of interest on a plant chromosome, and are widely used to study the organization of plant genomes and for the construction of genetic linkage maps. Molecular markers are independent from environmental variables and can be scored at any stage in the life cycle of a plant. Over the last several years, there has thus been marked increase in the application of molecular markers in the breeding programmes of various crop plants. Molecular markers not only facilitate the development of new varieties by reducing the time required for the detection of specific traits in progeny plants, but also fasten the identification of resistance genes and their corresponding molecular markers, thus accelerating efficient breeding of resistance traits into wheat cultivars by marker assisted selection (MAS). The availability of back-cross derived near isogenic lines (NILs) have also facilitated the analysis of various lines by using different marker systems. The introduction of alien genetic variation into wheat is a valuable and proven technique for wheat improvement. Wild relatives of wheat provide an enormous resource of new genes for wheat improvement, particularly disease and stress tolerance. These genes can be of use if recombined into lines adapted to the conditions of a particular region.

Initial studies on the application of molecular markers in wheat relied on the hybridisation based Restriction Fragment Length Polymorphism (RFLP) system. RFLP maps provided a more direct method for selecting desirable genes via their linkage to easily detectable markers thereby expediting the movement of desirable genes among varieties (Tanksley et al. 1989). The factors that had been instrumental for the use of RFLP in wheat was the limited

number of polymorphisms observed among wheat lines and more significantly the availability of aneuploid stocks for the determination of chromosomal location of genes. In wheat, RFLP's have been used to map seed storage protein loci (Dubcovsky et al. 1997), loci associated with flour colour (Parker et al. 1998), cultivar identification (Vaccino et al. 1993), vernalization (*Vrn1*) and frost resistance gene on chromosome 5A (Galiba et al. 1995), intrachromosomal mapping of genes for dwarfing (*Rht12*) and vernalization (*Vrn1*) (Korzun et al. 1997), resistance to preharvest sprouting (Anderson et al. 1993), quantitative trait loci (QTL's) controlling tissue culture response (*Tcr*) (Ben Amer et al. 1997), nematode resistance (Eastwood et al. 1994; Williams et al. 1996; Lagudah et al. 1997), milling yield (Parker et al. 1999), resistance to chlorosis induction by *Pyrenophora tritici-repentis* (Faris et al. 1997). RFLP markers are also useful in selection programs for resistance against pests and pathogens, which is otherwise labor and time consuming, and to detect homozygous individuals and have been used for resistance to barley yellow dwarf virus (Crasta et al. 2000), resistance to wheat spindle streak mosaic virus (Khan et al. 2000), resistance against powdery mildew (Hartl et al. 1993; Ma et al. 1994; Hartl et al. 1995), resistance against leaf rust (Dedryver et al. 1996; Autrique et al. 1995; Feuillet et al. 1995; Schachermayr et al. 1995; Schachermayr et al. 1994), resistance against cereal cyst nematode (Eastwood et al. 1994; Lagudah et al. 1997). The use of RFLP analysis in wheat has, however, been of limited use in the intervarietal analysis due to low level of polymorphism and the high cost for screening in breeding situations.

With the development of polymerase chain reaction (PCR) methodologies, Random Amplified Polymorphic DNA (RAPD) emerged as a convenient and effective technique for tracing alien chromosome segments in translocation lines (Williams et al. 1990; Devos and Gale, 1992). RAPD markers provide a useful alternative to RFLP analysis for screening markers linked to a single trait within near isogenic lines and bulked segregants. He et al. (1992) reported the development of a DNA polymorphism detection method by combining RAPD with DGGE (denaturing gradient gel electrophoresis) for pedigree analysis and fingerprinting of wheat cultivars. RAPD markers can be converted to more user-friendly Sequence Characterized Amplified Region (SCAR) markers, that display a less complex banding pattern. SCAR markers linked to resistance genes against fungal pathogens have been characterized in combination with RAPD and RFLP (Procunier et al. 1997; Myburg et al. 1998; Liu et al. 1999). In recent years, RAPD and other PCR based markers like Sequence Characterized Amplified Regions (SCAR), Sequence Tagged Sites (STS) and Differential Display

Reverse Transcriptase PCR (DDRT-PCR) are increasingly being used for identification of desirable traits in wheat and related genera. These markers have been used in particular for disease resistance against viral and fungal pathogens and also for insect and nematode pests and have the potential of pyramiding of resistance genes for effective breeding programs. PCR based markers have been extensively characterized for genes of resistance against common bunt, *Tilletia tritici*, (Demeke et al. 1996); powdery mildew, *Erysiphe graminis* (Hartl et al. 1995; Qi et al. 1996), leaf rust, *Puccinia recondita* (Dedryver et al. 1996; Feuillet et al. 1995; Seyfarth et al. 1999), resistance against Hessian fly, *Mayetiola destructor* (Dweikat et al. 1994) and Russian wheat aphid, *Diuraphis noxia* (Myburg et al. 1998; Venter and Botha, 2000).

Simple sequence repeats or microsatellites are more promising molecular markers for the identification and differentiation of genotypes within a species. The high level of polymorphism and easy handling has made microsatellites extremely useful for different applications in wheat breeding (Devos et al. 1995; Roder et al. 1995; Bryan et al. 1997; Korzun et al. 1997; Roy et al. 1999; Stachel et al. 2000). Microsatellites have also been used to identify resistance genes like *Pm6* from *Triticum timopheevii* (Tao et al. 1999) and *Yr15* from breadwheat (Chague et al. 1999). In near future, molecular markers can provide simultaneous and sequential selection of agronomically important genes in wheat breeding programs allowing screening for several agronomically important traits at early stages and effectively replace time consuming bioassays in early generation screens.

Genomics and future outlook

Genomics is the discovery and study of many genes simultaneously on a genome-wide scale. In the pregenomic era, gene discovery was the limiting factor for the improvement of crops by genetic engineering. For the upcoming postgenomic era, an exponential number of genes will be discovered through genome sequencing efforts on *Arabidopsis* and rice. Wheat genomes are large in comparison to the current plant models and are ideal targets for partial genome sequencing (Keller and Feuillet, 2000). However, due to a high level of genetic colinearity at a broader genetic level and also at the gene level, comparative genome analysis studies can serve as efficient tools for transferring information and resources from well studied genomes to the related genera (Devos and Gale, 1997; Schmidt, 2000). DNA mapping and sequence information generated in the rice genome project would be highly useful for the characterization of larger genomes like wheat. Molecular isolation of agronomically important genes and

an understanding of their action will contribute immensely to breeding of cultivars. With the availability of detailed information regarding the location and function of gene(s) encoding for useful traits, scientists in future will be well equipped for efficiently creating varieties with exact combinations of desirable traits. However, genetic transformation will remain a significantly important tool for understanding gene functions and for testing the utility of new sequences. In near future, crop varieties could be tailor-made to meet both local consumer preferences and the demands of particular niche or environment. The new tools of biotechnology not only have the potential for increasing the effectiveness and efficiency of wheat breeding programs, and but will also provide insights into the genetic control of key traits to be used for genetic manipulation. Application of biotechnology will thus contribute greatly to improving yield stability by generating plants with improved resistance to biotic and abiotic stresses rather than raising the overall yield. The coming years will undoubtedly witness an increasing application of biotechnology for the genetic improvement of wheat.

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Tables

Table 1. Selectable marker genes used in wheat transformation.

Gene	Selective agent	Mode of Action	Reference
<i>pat, bar</i>	Phosphinothricin, bialaphos	Inhibits glutamine synthase	Vasil et al. 1992 Vasil et al. 1993 Weeks et al. 1993
<i>nptII</i>	Kanamycin, G418, paromomycin	Binds 30S ribosomal subunit, inhibits translation	Nehra et al. 1994 Cheng et al. 1997 Witzens et al. 1998
<i>hpt</i>	Hygromycin	Binds 30S ribosomal subunit, inhibits translation	Ortiz et al. 1996
EPSPS oxidoreductase	Glyphosate	Inhibits aromatic acid biosynthesis (EPSPS)	Zhou et al. 1995
Glyphosate oxidoreductase (GOX)	Glyphosate	Degradation of glyphosate into aminomethyl phosphonic acid	Zhou et al. 1995
Mannose-6-phosphate Isomerase	Mannose	Ability to grow on mannose as a sole carbon source	Hansen and Wright, 1999
Cyanamide hydratase (<i>Cah</i>)	Cyanamide	Ability to grow on cyanamide containing media, converts cyanamide into urea	Weeks, 2000

Abbreviations:

bar and *pat*: encodes a phosphinothricin acetyl transferase

nptII: neomycin phosphotransferase II

hpt: hygromycin phosphotransferase

EPSPS: 5-enolpyruvyl-shikimate-3-phosphate synthase

Table 2. Wheat transformation by particle bombardment.

Reference	Target tissue	Promoter/reporter	Selectable marker	Comment
Wang et al. 1988	Cell suspension	<i>Adh1/gus</i>	-	Transient expression
Oard et al. 1990	Leaf base Apical tissue	<i>Adh1/gus</i> <i>35S/ gus</i>	-	Transient expression (air gun apparatus)
Chibbar et al. 1991	Immature embryo	<i>35S/cat</i> <i>35S/gus</i>	-	Transient expression
Daniell et al. 1991	Leaves, Immature embryo or Anther derived calli	<i>35S/ gus Double</i> <i>psbA/gus</i>	<i>nptII</i> -	Transient expression of the gus gene. First report of foreign gene expression in a monocot chloroplast
Shimada et al. 1991	Pollen embryos	<i>35S/gus</i>	-	Transient expression
Vasil et al. 1991	Cell suspension	<i>E35S/gus</i>	<i>nptII, EPSPS</i>	Generation of stable transformed callus lines expressing the marker genes
Vasil et al. 1992	Embryogenic callus (5-7 month old)	<i>Adh1/gus</i>	<i>bar</i>	Stable transformation, production of basta resistant fertile transgenic plants.
Perl et al. 1992	Scutellar calli	<i>Act1/gus</i>	-	Enhanced transient expression was observed when silver thiosulphate and $\text{Ca}(\text{NO}_3)_2$ was used in place of CaCl_2
Vasil et al. 1993	Immature embryo, embryogenic calli (1-2 month old)	<i>Adh1/gus</i> <i>Ubi/gus</i> <i>35S/ gus</i>	<i>bar</i>	Stable transformation. Generation of flowering transgenic plant in 7-9 months
Weeks et al. 1993	Callus derived from immature embryo	<i>Ubi/gus</i>	<i>bar</i>	Stable transformation producing nine independent lines of fertile transgenic wheat
Becker et al. 1994	Scutellar tissue of immature embryos	<i>Act1/gus</i>	<i>bar</i>	Stable transformation and Mendelian segregation of the transgene
Iglesias et al.1994	Embryonic meristem	<i>Act1/gus</i> <i>35S/gus</i>	-	Transient expression in meristem cells, which is 10 fold less than the surrounding tissues(microtargetting).
Takumi et al. 1994	Cell lines	<i>35S/gus</i> <i>35S-35S/gus</i> <i>Adh /gus</i> <i>Act1/gus</i>	-	Highest level of transient expression was observed by rice <i>Act1</i> promoter among all the promoter/intron combinations tested
Loeb and Reynolds,1994	Microspore-derived embryoids	<i>Ubi/gus</i>	<i>bar</i>	Transient expression, detection of GUS expression in haploid plants 5 weeks post-bombardment.
Nehra et al. 1994	Isolated scutella, Scutellum callus	<i>35S/gus</i> <i>Act1/gus</i>	<i>bar, nptII</i>	Stable transformation and mendelian inheritance of the transgenes.

Zhou et al. 1995	Immature embryo	35S (duplicated) <i>Ubi1</i>	CP4, GOX	Stable transformation of wheat. The successful use of glyphosate tolerant CP4 and GOX genes as selectable markers.
Altpeter et al. 1996a	Immature embryo	<i>Ubi1/gus</i>	<i>bar</i>	Accelerated production of transgenic wheat (8-9 weeks time for generation of transgenic plants)
Altpeter et al. 1996b	Immature embryo	<i>Ubi1/gus</i>	<i>bar</i>	Integration and expression of high molecular weight (HMW) glutenin subunit 1Ax1 in wheat
Blechl and Anderson, 1996	Immature embryo	<i>Dy10</i>	<i>bar</i>	Transformation of wheat with a modified HMW glutenin subunit gene under native regulatory elements
Ortiz et al.1996	Immature embryo calli	35S/ <i>gus</i>	<i>hpt, bar,</i>	Hygromycin resistance as an efficient selctable marker for wheat stable transformation
Takumi and Shimada, 1996	Immature scutellum tissue	35S/ <i>gus</i> <i>Act1/gus</i>	<i>bar</i>	Successful cotransformation of the <i>gus</i> and <i>bar</i> genes . The authors reported the influence of culture duration on the transformation efficiency of the scutellum tissue
De Block et al. 1997	Embryogenic calli	35S	<i>bar</i>	Engineering of nuclear male sterility in wheat by introduction of the <i>barnase</i> gene under the control of tapetum-specific promoter
Lonsdale et al. 1998	Scutellar calli	<i>Ubi1/luciferase</i>	<i>bar</i>	Stable transformation of wheat by employing luciferase as a reporter gene to identify the transition from transient to stable expression
Barro et al. 1998	Scutellar tissue	<i>Ubi/gus</i> <i>Act1/gus</i> 35S	<i>bar</i> <i>nptII</i>	Low copy number and simple integration patterns in transgenic lines. This report investigated on the influence of auxins on the transformation of wheat
Leckband and Lorz, 1998	Immature embryos	<i>Vst1/gus</i> 35S	<i>pat</i>	Transformation of a stilbene synthase gene of <i>Vitis vinifera</i> (<i>Vst1</i>) for increased fungal resistance
Witzens et al. 1998	Cultured immature embryo	<i>Act1/gus</i>	<i>Ubi/ bar nptII</i>	Generation of stably transformed plants with <i>bar</i> and <i>nptII</i> as the selectable marker. No transgenic plant was obtained with the <i>hpt</i> gene
Altpeter et al. 1999	Immature embryos	<i>Ubi</i>	<i>bar</i>	Transformation of a barley trypsin inhibitor gene into wheat for increased resistance to insects
Bliffeld et al. 1999	Isolated scutella	<i>Act1 Ubi1</i>	<i>bar</i>	Transformation of a barley seed chitinase gene into wheat for incresed resistance to powdery mildew disease.
Chawla et al. 1999a; Chawla et al. 1999b	Scutellum	35S/ (<i>Bperu</i> and <i>C1</i>) <i>Act1</i>	<i>bar</i>	Use of anthocyanin regulatory genes as a reporter for stable transformation of wheat
Chen et al. 1999	Immature embryos	<i>Ubi</i> 35S	<i>hpt</i>	Transformation of a rice thaumatin like protein into wheat for enhanced resistance to scab caused by <i>Fusarium</i> .
Harvey et al. 1999	Scutellar calli	<i>Ubi1/luciferase</i>	<i>bar</i>	Use of tissue culture regenerated plants as a source of explants resulted in a significant increase in the transformation efficiency

Ingram et al. 1999	Microspore-derived embryo	<i>Ubi/gus</i>	<i>bar</i>	Transient expression
Srivastava et al. 1999	Immature embryos	<i>Act1 Ubi1</i>	<i>bar</i>	Generation of single copy transgenic wheat based on the use of a strategy based on site-specific recombination of Cre-lox system
Stoger et al. 1999	Immature embryos	<i>Rss1 Ubi1</i>	<i>bar</i>	Expression of the insecticidal lectin from <i>Galanthus nivalis</i> in transgenic wheat plants for increased insect resistance
Takumi et al. 1999	Immature embryo	<i>35S Act1 Ubi</i>	<i>bar</i>	Development of transposon tagging system in wheat by transformation of maize activator (Ac) element and transformation of dissociation (Ds) element in transgenic lines expressing Ac.
Uze et al. 1999	Cultured immature embryo	<i>Ubi/gus</i>	<i>Ubi/bar</i>	Highest transformation efficiencies was obtained with linearized single stranded and double stranded plasmids
Bieri et al. 2000	Scutellum derived calli	<i>Act1</i>	<i>bar</i>	Introduction of a barley seed ribosome-inactivating protein for enhanced fungal resistance
Jordan, 2000	Immature embryos	<i>Act1/gfp 35S</i>	<i>nptII</i>	Green fluorescent protein as a visual marker for wheat transformation
Sivamani et al. 2000	Immature embryos	<i>Ubi</i>	<i>bar</i>	Transformation of a barley gene (<i>HVA1</i>) encoding a late embryogenesis abundant protein into wheat for tolerance to drought stress
Zhang et al. 2000	Immature embryos	<i>Ubi</i>	<i>bar</i>	Stable transformation and expression of <i>bar</i> and the bacterial ribonuclease gene.

Abbreviations:

Act1: rice actin promoter

Adh1: alcohol dehydrogenase 1 promoter

bar and pat: encodes a phosphinothricin acetyl transferase

Bperu and C1: regulatory elements of anthocyanin biosynthesis

cat: chloramphenicol acetyl transferase

Dy10: an high molecular weight glutenin subunit (HMW-GS) gene sequence containing the promoter and start site

gus: encodes β -glucuronidase

gfp: green fluorescent protein

hpt: encodes hygromycin phosphotransferase

nptII: encodes neomycin phosphotransferase

psbA: promoter fragment from pea chloroplast genome

35S: Cauliflower Mosaic Virus (CaMV) 35S promoter

E35S: enhanced 35S promoter

Ubi1: maize ubiquitin promoter

Rss1: Rice sucrose synthase 1 promoter

Vst1: promoter of Vitis stilbene synthase gen

Table 3. *Agrobacterium*-mediated transformation of wheat.

Reference	Inoculum	Explant	Comment
Woolston et al. 1988	C58na ^R (pJIT31 and 1-4d old seedlings Seedlings direct injection Wooiston et al, 1988 pJIT33, cloned wheat dwarf virus (WDV) DNA)	1-4 d-old seedling	Direct injection of <i>Agrobacterium</i> inoculum to the basal portion of wheat seedlings. Successful infection of wheat seedlings.
Dale et al. 1989	C58 (pBin19 with a tandem dimer of WDF)	Embryos, 4-d-old <i>in vitro</i> grown seedlings	Use of different inoculation procedures, wounding, vacuum infiltration and direct injection. <i>Agrobacterium</i> was found to deliver wheat dwarf virus DNA to wheat.
Marks et al. 1989	<i>A. tumefaciens</i> and <i>A. rhizogenes</i> (15 strains) [pJIT33 (cloned WDV DNA)]	4-d-old wheat seedlings	<i>A. rhizogenes</i> strains were found to give a higher proportion of agroinfected plants than <i>A. tumefaciens</i> strains. superiority of nopaline strains of <i>A. tumefaciens</i> over octopine strains.
Hess et al. 1990	C58C1pGV3850 ::1103neo	Wheat florets	Pipetting of <i>Agrobacterium</i> into the spikelet of wheat. Generation of fertile southern positive plants by the contact of bacteria and pollen
Mooney et al. 1991 Mooney and Goodwin, 1991	C58C pGV3850::103neo	Immature embryos	Demonstrated the wound independent <i>in vitro</i> attachment of <i>Agrobacterium</i> to wheat embryos. Wounding caused by mechanical and enzymatic treatments promoted the adherence of the bacteria at the wound site.
Chen and Dale, 1992	<i>A. tumefaciens</i> disC58/pJIT33, disarmed C58, <i>A. rhizogenes</i> LBA9402(pJIT33)	Apical meristems of dry wheat seeds	Incubation of exposed apical meristems of dry wheat seeds with <i>Agrobacterium</i> . High frequency of infection.
Zaghmout and Trolinder, 1993	A281 (pKIWI105), LBA4404 (pKIWI105)	Intact cells of slowly growing embryogenic callus	Reported the transient <i>gus</i> gene expression by electroporation
Mahalakshmi and Khurana, 1995	A348 (pMON20), A281 (pBI121), GV2260 (p35SGUSINT)	Leaf bases, callus, mature seeds, punctured seedling	Suitability of various explants and <i>vir</i> gene induction and the effect of mechanical wounding was assessed by abrasing seed explants with a sand paper.
Cheng et al. 1997	C58 (pMON18365)	Immature embryos, Embryogenic calli	<i>Agrobacterium</i> mediated stable transformation, with Mendelian inheritance of the transgene to the T ₁ generation.
McCormac et al. 1998	EHA101, LBA4404, pBECKS.red, - pBECKS.GUSintron, pBECKS.sgfp-S65T, pBECKS ₄₀₀ .Zsk	Immature embryos and immature embryo derived calli	Use of cell-autonomous reporter genes (anthocyanin biosynthesis regulatory genes, <i>gus b</i> -glucuronidase), synthetic grren fluorescent protein (<i>gfp</i>) for studying the localization of transformation events.
Guang-Min and Zhong-Yi, 1999	AGL25(p ^{unn2})	Immature embryos and immature embryo derived calli	Stable transformation of wheat.
Mi et al. 2000	LBA4404 (pCAMBAR Ubi-TLP)	Precultured immature embryos	Introduction of rice thaumatin like protein (TLP) gene

Amoah et al. 2001	AGL1 (pAL156)	Immature inflorescence	Study of various factors influencing transient expression of <i>gus</i> gene
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Table 4. Genetic transformation of wheat for pathogen and pest resistance.

Gene	Source	Mode of Action	Construct (Promoter)	Comment/Reference
Coat Protein	Barley yellow mosaic virus	Coat protein-mediated resistance	pEmuPAT-cp (35S)	Karunaratne et al. 1996
Ribosome Inactivating protein (RIP)	<i>Hordeum vulgare</i>	Specific glycosidases that remove a conserved adenine residue from the large rRNA of the large ribosomal subunit	pRipChi (Ubi)	Interfere with normal plant regeneration and development (Bliffeld et al. 1999)
		Inactivates ribosome and blocks translation elongation	<i>I-Sec1</i> (35S-RTBV intron)	Moderate/no protection against <i>Erysiphe graminis</i> (Bieri et al. 2000)
Chitinase	<i>Oryza sativa</i>	Cell wall degradative enzyme Act on cell wall polysaccharide, chitin	pAHG11 Chi11_Ubi pro-bar-nos	Lack of transgene expression (Chen et al. 1998; Chen et al. 1999)
	<i>Hordeum vulgare</i>		Ubi	Increased resistance to infection by <i>Erysiphe graminis</i> (Bliffeld et al. 1999)
Thaumatococin like Protein (<i>tlp</i>)	<i>Oryza sativa</i>	Alter membrane permeability and/or Cellular signal transduction cascades	pGL2ubi-tlp (Ubi/ <i>tlp</i> /CaMV 35S/hpt)	Enhanced resistance against <i>Fusarium graminearum</i> in T ₁ , T ₂ and T ₃ plants (Chen et al. 1999)
Stilbene synthase	<i>Vitis vinifera</i>	Phytoalexin synthesis Synthesizes the phytoalexin trans-reveratrol	pGBI, pGBII (Vst1)	Induction of stilbene synthase mRNA after wounding and infection in T ₁ and T ₂ plants (Leckband and Lorz, 1998)
Killer protein (KP)	<i>Ustilago maydis</i> infecting virus	Virally encoded antifungal protein. Inhibit growth of sensitive cells	pUbi:KP4 (Ubi)	Increased endogenous resistance against <i>Tilletia tritici</i> (Clausen et al. 2000)
Lectin	<i>Galanthus nivalis</i> agglutinin (GNA)	Binding to the insect gut surface	pRSsGNA (<i>Rss1</i>) pUbiGNA (Ubi)	Decreases the fecundity, but not the survival of the grain aphid <i>Sitobion avenae</i> (Stoger et al. 1999)

Proteinase Inhibitor	<i>Hordeum vulgare</i> (Barley trypsin inhibitor-CMe)	Regulators of endogenous proteinases	C-Me (BTI-CMe)	Inhibition of early insect larvae in transgenic seeds;
		Lower protease activity in insect guts leading to direct shortage of amino acids	pUPMBI-66 (Ubi)	No significant effect on leaf feeding insects (Altpeiter et al. 1999)
	<i>Sagittaria trifolia</i> [arrowhead proteinase inhibitor (API)]	Serine proteinase inhibitor	pBIAH-A(B)	Introduction of arrowhead proteinase inhibitor genes into wheat genome by pollen tube pathway (Mu et al. 1999)

Abbreviations:

bar and pat: encodes a phosphinothricin acetyl transferase

gus: encodes β -glucuronidase

hpt: encodes hygromycin phosphotransferase

35S: Cauliflower Mosaic Virus (CaMV) 35S promoter

Ubi1: maize ubiquitin promoter

Rss1: Rice sucrose synthase 1 promoter

Vst1: promoter of *Vitis* stilbene synthase gene

RTBV: rice tungro bacilliform virus