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Genetic Transformation of Rice: Problems, Progress and Prospects

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Abstract

Plant genetic engineering has become one of the most important molecular tools in the modern molecular breeding of crops. Now a days, production of transgenic plants is a routine process in some crop species. Transgenes are delivered into plants to confer novel traits such as improving nutritional qualities, resistance to pests. It is possible to insert genes from plants at evolutionary distant from the host plant, as well as from fungi, viruses, bacteria and even animals. Genetic transformation requires penetration of the transgene through the plant cell wall, facilitated by biological or physical methods. Over the last few decades, a significant progress has been made in the development of new and efficient transformation methods. Despite a variety of available DNA delivery methods, Agrobacterium and Biolistic mediated transformation remain two predominantly applied approaches. The objective of this article is to review the currently used methods for genetic plant transformation, their biological requirements and critical parameters.

Keywords: Genetic transformation rice; *Agrobacterim*; Biolistic transformation.

Introduction

Cereals are the most important source of calories to humans since rice, wheat and maize provide 23%, 17% and 10% calories globally [1]. Rice (*Oryza sativa* L.) is a well-known economical cereal, because it is a staple food included in the diet and most important source of food for more than half of the population. It has been reported that more than 90 per cent of the world's rice is grown and consumed in Asia, where it is a major source of income to rural people [2,3]. Adoption of green revolution varieties led to a drastic change in rice production. Between 1996 and 2011, the population of the low-income countries grew by 110 per cent, but rice production increased by 180 percent only from 257 million tons in 1996 to 718 million tons in 2011. Despite these advances in rice production, still 800 million people is not getting food every day. It is estimated to increase up to 25 per cent of rice production by the year 2030 [1]. This additional rice must be produced from good lands without opening up more fragile lands for rice cultivation. Agriculture production is decreasing due to biotic and abiotic stresses. The major abiotic stresses worldwide which cause threat to food security are high salinity, drought, submergence and cold [4-8]. Among these abiotic stresses, salinity is one of the major factors restricting productivity of crop plant worldwide [9]. Area under salt stress is increasing due to certain factors like climate change, rise in sea levels, excess irrigation without proper drainage in islands and underlying rocks rich in harmful salts,. If current scenario will continue, 50 per cent of present cultivated land will be lost for agriculture by 2050 [10]. Therefore, abiotic stresses are one of the main concerns to fulfill the required food demand [11]. Rendering the problem will entail development of rice varieties, which have higher yields, excellent grain quality, and resistance to biotic and abiotic stresses.

Many breeding programs have been conducted to increase the environmental stress tolerance, pest and disease resistance to the crop. Although conventional breeding programs such as Conventional hybridization, hybrid breeding, wide hybridization and ideo type breeding have developed some rice varieties in addition to several lines have been released in the Philippines, Bangladesh and India [12] but the success rate of conventional breeding is not sufficient [12,13] to meet the requirements. So there is a need to develop rice varieties that can withstand high levels of salt, drought and water stress with optimal yield levels.

Therefore, it is of utmost important to augment the productivity of rice to cope with the increased threat of population boom which is expected to reach 9.1 billion by 2050. To combat these issues, various methods of conventional breeding programs have been deployed, albeit with little progress. In this aspect, genetic transformation can be integral tool in breeding strategies. It permits access to an unlimited gene pool through the transfer of desirable genes [14]. The development of plant transformation techniques during the past decades has made it possible to improve crop plants by introduction of cloned genes. The two most critical steps to be the master for transformation of plants are to transfer of foreign DNA into the plant cell and regeneration of plants from transformed cells [15]. The callus induction and regeneration in tissue culture of rice depend upon different factors like genotype, type of explants and media supplement like basal salts, organic component and growth regulator [16-19]. Development of genetically engineered plants with enhanced tolerance to abiotic and biotic stresses is the important challenges in plant biotechnology research. Rice transformation is a major goal in cereal biotechnology, because rice is world's most important food crop and it is also known as model of cereal genomics [20].

In early 1980s, genetic transformation of crop plants i.e. based on recombinant DNA technology was started and this offered an advantage of transferring novel genes across taxonomic boundaries unlike conventional breeding [3]. Genetic engineering has been used as a prominent tool for rice improvement. Although gene transformation in *japonica* rice is performed routinely in several laboratories, but the system in *indica* rice is more complicated [15]. Until now, the number of copies of a gene(s) inserted and chromosomal locations of the integrated genes are not controllable, the expression of the introduced

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genes varies among individual transformants. Therefore, a relatively large number of transgenic plants must be developed in order to select desirable transformants as well as to study the expression of introduced genes [21].

The most commonly used method for transformation are Biolistic approach and Agrobacterium mediated transformation. This review will summarise various gene delivery methods applied to improve rice traits. Subsequent molecular analysis of the transgenic rice will also be discussed. Additionally, it will consider the future prospects of transgenic researches on the crop.

Genetic Transformation

Purpose of genetic transformation

Main purpose of genetic transformation is to generate plants with useful phenotypes i.e. unachievable by conventional plant breeding, to correct faults in cultivars more efficiently than conventional breeding and to allow the commercial value of improved plant lines to be captured by those investing in the research more fully than is possible under intellectual property laws governing conventionally bred plants. Some reasons for genetic modifications are yield improvement, more resistant to disease and pest resistance, herbicides tolerance, better nutritional value, increased shelf life, better climatic survival by increasing tolerance to drought, flood or frosty conditions to allow the use of previously inhospitable land, higher crop yields, reduced farm costs, increased farm profit and improvement in health and environment..

Biological requirements for transformation

The essential requirements in a gene transfer system for production of transgenic plants are availability of a target tissue including cells competent for plant regeneration, a method to introduce DNA into those re-generable cells and a procedure to select and to re-generate transformed plants at a satisfactory frequency.

Methods of Genetic Transformation

Agrobacterium mediated genetic transformation

The soil pathogen *Agrobacterim tumefaciens* has been extensively studied since 1907, when it was identified as the causative agent of crown gall disease [22-24] Braun initially proposed the Agrobacterium as a source of a 'tumor inducing principle', possibly DNA, that permanently transformed plant cells from a state of quiescence to active cell division.

A. tumefaciens is a soil dwelling bacteria that naturally infect dicots and causes tumorous growth resulting in crown gall disease. Tumor formation results from incorporation of T-DNA (transfer DNA), a part of small independent DNA molecule outside the bacterial genome called Ti (tumor inducing) plasmid. Phenolic compounds exuded from plant wounds that stimulate the expression of *vir* genes, located on Ti plasmid and responsible for its excision, transfer and integration into plant genome. The natural capability of *Agrobacterium* was manipulated in plant transformation by replacing the genes causing tumorous growth by genes of interest [25]. However, *Agrobacterium* has a natural tendency to infect dicot plants, whereas, monocots including wheat were considered recalcitrant to *Agrobacterium* transformation. Therefore, most of the *Agrobacterium*-mediated transformation procedures were established for dicots, whereas, monocots including important cereals such as wheat, rice and maize lagged behind for a considerable time [26-28]. Low frequency of T-DNA transfer into the target genome was the major limitation. Nonetheless improvements of co-cultivation conditions, use of acetosyringone, selection and regeneration methods for transformed tissues and incorporation of super binary vectors have helped in extending the host range of *Agrobacterium* to several monocots including important cereals.

Agrobacterium approach is the most popular method to deliver genes to plant cells because of its clean insertion, low-copy number of the inserted genes, easy to handle, higher efficiency, more predictable pattern of foreign DNA integration. Studies on *A. tumefaciens* provided the basis that has made this soil bacterium as dominant customer for plant transformation.

Critical parameters to increase the efficiency of *Agrobacterium*-mediated genetic transformation are: Concentration of *Agrobacterium* broth (in terms of OD), time of inoculation, time of co-cultivation, concentration of acetosyringone.

Optical density of *Agrobacterium* culture must be in the range of 0.4 to 1.00. During the *Agrobacterium* infection, OD_{600} of the bacterial suspension must be adjusted within 1.0 because high concentrations of bacteria caused serious injury to callus thus lowering the transformation efficiency.

Co-cultivation duration (5-25min), exposure the explants to 0.4 OD Agrobacterium for 10 min was found to be optimum.

Co-cultivation period should be between 2 to 5 days. Co-cultivation for 2 days showed better infection without browning or *Agrobacterium* overgrowth.

Co-cultivation with filter paper increased transformation frequency because the filter paper prevented overgrowth of bacteria. In co-cultivation medium, use of cysteine reduces browning of the calli, and Acetosyringone as well as glucose to induce the vir gene activity. Concentration of acetosyringone should be between 100-150 μM. But higher concentration of acetosyringone (200 μM) in co-cultivation media resulted in lower frequency of regeneration likely due to necrotic effect of acetosyringone.

Biolistic Process (Particle Bombardment) mediated genetic transformation

Biolistic transformation was initially invented in 1987 by American Geneticist, Associate Professor John C, Sanford from Cornell University where he and his colleagues showed that small micro projectiles could be delivered into a cell without killing it. In this approach (Also called gene gun or particle bombardment), microscopic particles coated with the DNA fragment representing the desired gene are shot into the plant cells using a special device called gene gun. The key principle behind the gun is to accelerate particles by explosion. A small portion of the DNA which enters the cells becomes incorporated into the chromosomes of the plant cell. The gene gun technique helps to overcome some of the deficiencies of *Agrobacterium* method such as bacterial contamination, low-efficiency of transfer to cereal crops, and inconsistency of results. The tungsten was first used or DNA coating later on it was replaced by gold particles because tungsten could be oxidized easily and it was potentially harmful to the cell.

It has many advantages than other techniques like rapid gene transfer, efficient, non-specific to tissue, complex cloning strategies with no biological constraints or host limitations and simultaneous multiple gene transfer. There are no intrinsic vector requirements

so transgenes of any size and arrangement can be introduced, and multiple gene co-transformation is straightforward. It has bigger advantage that the delivered DNA can be manipulated to influence the quality and structure of the resultant transgene loci. This approach can be used for transfer of more than one gene simultaneously in a host plant. As many as 14 genes have been co-introduced in rice by this approach [29]. Nowadays, particle bombardment is the most efficient way to achieve plastid transformation in plants and is the only method so far used to achieve mitochondrial transformation [30]. A genotype independent method for rice transformation was originally reported by Christou et al. [31] and it has been widely used throughout the world. Researchers at the International Rice Research Institute, Philippines, have used particle bombardment successfully to transform over 20 different cultivars adapted to different eco-geographic conditions. These cultivars have been transformed with a range of agronomically important genes like *psy, crt1, cry, ferritin, FRO2, Xa21, Bt, Chitinase,*

Genotype	Explants	Promoters	Strain	Plasmid	Transgene	Marker gene	Transformation Efficiency	Transgenic Analysis	References
Ratna (IET411)	Seeds	35S	LBA4404	pCAMBIA1301	hpt	Kanamycin GUS	47%	PCR Analysis	Basu et al. [33]
IRGA424	Seeds	Mpi ubi	LBA4404	pX2.H.C1mpi Cry1Bgene pC1300.ubi Cry1B.nos	Cry1Aa Cry1B	GFP hph	86.4%	PCR Analysis	Pinto et al. [34]
IR36	Mature seeds	35S	EHA105AL	pCAMBL 1301	hpt	Uid A	98% callus induction	PCR Analysis	Krishnan et al. [35]
Indica rice	Mature seeds	35S	LBA4404	pKhg4	Cry1 Ac	Hygromycin resistance gene	-	-	Guruprasad et al. [36]
IR64 Swarna CSR10 PB1	Mature dry seeds	35S	LBA4404	pCAMBL A1304	Glyoxylase 1 (Bigly1)	nptII hptII uidA GFP	Approximately 45%	PCR Analysis	Sahoo et al. [37]
Bg 250	Mature seeds	35S	GV3101	pCAMBL 1303	hptIV	Hygromycin GUS	20%	GUS Analysis	Ratnayaka et al. [38]
Pusa Basmati 1	Mature seeds	35S	LBA4404	pCAMBIA 1301	Am-SOD	Kanamycin resistance genes Hygromycin resistance genes	Very good	PCR Analysis Southern blotting	Sarangi et al. [39]
Kalizira Radhunipagol Tulsimala Pusa basmati-1	Mature embryo with endosperm	35S	EHA105	pIG121-Hm	Uid hpt	nptII	40-75%	GUS assay	Hossain et al. [40]
Heugnam—byeo Daesanbyeo	Scutellum of mature rice seeds	OsCc1 35S	LBA4404	pMJC-GB pMJC-GH	Isoflavone synthase 2 Chalcone reductase	bar hpt	12.8% (Hygromycin At Callus proliferation stage And Phosphinothricin At Shoot regeneration stage) 100% (for vice versa)	RT-PCR	Sohn et al. [41]
HKR-46 HKR-126	Seeds	EHA105		pCAMBIA 1301	Uid Hygromycin resistance gene Carbenicillin resistance gene	Hygromycin resistance gene Carbenicillin resistance gene	28.9%- 44.4%	GUS Assay	Saharan et al. [42]
Taipei 309	Mature zygotic embroys	Glutelin 35S	LBA4404	pAGt1Fe pAGt1Me	Ferritin (pte) Metallathionein-like (rgMT) Phytase (phyA)	hptII hptIV		Western blot Northern blot	Lucca et al. [43]
Senia Tebre Bahia	Mature seeds		LBA4404	pTOK233	GUS gene	hpt npt	5% (Tebra) 3% (Bahia, Senia)	Southern blot	Pons et al. [44]
Taipei 309 Pusa basmati 1 Tinawen	EC		LBA4404	pTOK233		hpt		16-31%(Taipei309) 12-21%(Pusa basmati1) 10-19%(Tinawen)	Azhakanandam et al. [45]

Basmati 122 Tulsi Vaidehi	EC		LBA4404 A281	pNO1		hpt	4.3-5.5%		Datta et al. [46]
Jarah Amoroo	EC		EHA101 AGLO AGL1	p1G121-Hm pTO134 pWBVec10a pBS360 Pbs366		Hpt bar	>10%(Jarah) 2.8%(Amaroo)		Upadhaya et al. [47]
DS20, OMCS96, OMCS97, IR72, IR64	Mature seeds	35S Ubiquitin	LBA4404	PSBbarB-Ubicore And Psb35L-Hyg-L-Gus	barB Cre Hyg gusA	Nos	1.78-13.33%	Southern blot	Hoa et al. [48]
R321	SM		A2260	pGTGUSBAR		bar	15-30% for PPT resistant calluses		Enriquez-Obregon et al. [49]
Pusa basmati	EC		LBA4404	pTOK233		hpt	25%		Mohanty et al. [50]
Seven elite lines including koshihikari	EC		EHA101	P1G12Hm pGFPKH		hpt	25-34%		Hashizume et al. [51]
Tapei 309	EC SDS		LBA4404	pTOK233		hpt	2.3-3.6%		Khanna and Raina [52]
IR64 Karnal local	EC SDS		AGL1	pCAMIAI301		hpt	2.3-3.6%		Khanna and Raina [52]
Nipponbare Zhong8215 ZAU16 91RM T8340 Pin92-528 T90502 Kaybonnet	Mature Or Immature embroys	LBA4404 EHA105	35S Bp10 Ubioquitin	PkUB pKUC pKSB pKBB	Cri 1Ab Cry1Ac	GUS	52-100%	DNA and RNA blot Analysis	Cheng et al. [53]
E-yi105 E-Wan5ZSWG Pusa basmati Tapei 309	EC PCIE		 LBA4404	pTOK233 pTOK233 pBin93SΩGusint p1G12Hm		Hpt hpt	13.5% (Pusa basmati) 9.1-13% (E-yi105 and E-Wan5ZSWG) A few events		Zhang et al. [54] Uze et al. [55]
Nipponbare Kitaake	Mature seeds	EHA101	Nopaline synthetase Pubi-1	EHA101	PIG121Hm pSMABuba	Hygromycin resistance gene bialaphos resistance gene			Toki et al. [56]
Basmati 370 Basmati 385	Calli derived from scutella	35S	EHA101		hpt gus	Hygromycin			Rashid et al. [57]
Tapei 309	Calli derived from scutella	35S	LBA4404 EHA101		Hpt gus	Hygromycin			Deng et al. [58]
Gulfmont Jefferson Radon	Immature embroys	35S	LBA4404		Hpt gus	Hygromycin			Aldemita and Hodges [59]
TCs10 IR72 Maybelle	Isolated shoot apices	Actin 1	EHA101		bar	PPT			Park et al. [60]
Radon TCS10 IR72 Taipei 309	FIIE EC		LBA4404 At656 LBA4404	pTOK233 pCNL56 pTOK233		hpt hpt	27% (Radon) 1-5% (TCS10 and IR72)		Aldemita and Hodges [59]
Gulfmont Jefferson	EC		EHA101	PIG121Hm		hpt	8.3-13.3%		Dong et al. [58]
Maybelle	ISA		EHA101	pGSFR781 pBARNPT		bar	low		Park et al. [60]
Tsukinohikari Asanohikari Koshihikari	Calli derived from scutella, immature embroys, suspension cells	35S	LBA4404		Hpt gus	Hygromycin		Southern Analysis	Hiei et al. [61]

Tsukinahikari Asanohikari Kashihikari	EC FIIE SC		LBA4404 EHA101	pTOK233 pG121Hm		hpt	12.8-28.6%		Hiei et al. [61]
Shoot apices, root segments from young seedlings,scutella, immatureembroys, call, cells from suspension cultures	Tuskinikari Asanohikari Koshihikari	EHA101 LBA4404	p1G121Hm pTOK233	hpt	GUS Hygromycin	Gus assay	18.2-28.6% (Tuskinikari) 12.8-19.5% (Asanohikari) 17.6-18.8% (Koshihikari)		Hiei et al. [61]
Tannung 62	Precultured immature embroys		A281	pAG8			nptII	A few events	Chan et al. [62]
Taichung native 1	Roots from germinate seeds	Nos 35S	C58C1 LBA4404		nptII gus	G418		Southern Analysis	Chan et al. [62]
Nipponbare Fujisaka 5	Mature embryo from germinate seeds	Nos 35S	LBA4404 A281 A856		NptII Gus onco genes	Kanamycin		Southern Analysis	Raineri et al. [62]
Tainung 62	Cultured immature embroys	nos alpha-amy8	A281		nptII gus	G418			Chan et al. [62]

Table 1: Trends in rice (*Oryza sativa* L.) transformation through Agrobacterium-mediated genetic transformation.

enod12, PEPC, glgC, rolC, sd1 confirming the genotype independence of the transformation method (For details see Table 1) [32]. The main disadvantages of particle bombardment in comparison with *Agrobacterium* i.e. the tendency to generate large transgene arrays containing rearranged and broken transgene copies, are not borne out by the recent detailed structural analysis of transgene loci produced by each of the methods.

Genetic transformation occurs in the two stages: DNA transfer into the cell followed by DNA integration into the genome. The integration stage is much less efficient than the DNA transfer stage, with result of that only a small proportion of the cells that initially receive DNA actually become stably transformed. In many cells, DNA enters but expressed for a short time (transient expression), but it is never integrated and it is eventually degraded by nucleases. Transient expression occurs almost immediately after gene transfer, it does not require the regeneration of whole plants, and it occurs at a much higher frequency than stable integration. Therefore, transient expression can be used as a rapid assay to evaluate the efficiency of direct DNA transfer and to verify the function of expression constructs. In addition, transient expression following particle bombardment with a reporter gene such as gusA or gfp is used routinely to compare different expression constructs and to identify those with most appropriate activities. When the aim is to extract recombinant proteins from transgenic plants, transient expression following particle bombardment may also be used to produce small amounts of protein rapidly for testing [63]. Hoffman et al. [64] used particle bombardment for the mechanical transmission of poleroviruses and particle bombardment is routinely employed for the inoculation of whole plants and leaf tissues with viruses that are difficult to introduce via conventional mechanical infection. It also has an important role in extending virus-induced gene silencing (VIGS) into economically important crop plants [65].

Critical parameters to increase transformation efficiency

Type/physiological and developmental stage of the target tissue: Embryogenic calli of rice have been extensively used in particle bombardment experiments. The calli should be young not more than 30 days old. If we use old calli, the transformation efficiency will be low as calli loses its regeneration capacity ultimately affecting transformation efficiency.

Particle type/size, helium pressure and target distance on bombardment: Efficiency of the DNA delivery to target cells depends largely upon the size of micro-particles and the force in which these DNA coated particles are pushed towards the target cells. The depth of penetration into the target tissues can be controlled by varying particle size, helium pressure and the target distance. The target distance has direct relation with target area i.e. lesser the target distance narrow is the target area and greater the target distance, wider is the target area. So these three parameters are important in determining optimal conditions for efficient DNA delivery to target cells/tissues.

Effect of particle and DNA loads: For obtaining high efficiency of DNA delivery to target tissues, different DNA to particle ratios has been used to determine a suitable combination. A particle load of 3 mg (0.5 mg/shot) is sufficient to produce high level of transient GUS expression during conjunction by 9 µg of DNA (1:3 particle-DNA ratio).

In-Planta transformation

A number of advances in reducing the dependence to tissue culture have been made in rice transformation including, *in planta* transformation method. Cereal transformation via the tissue culture phase has been successful, but involves several limitations. The use of tissue culture allows selection of single transformed cells which are

regenerated in a whole plant. However, the tissue culture approach causes somaclonal variation due to both epigenetic effects and chromosomal rearrangements [66,67]. The *in planta* transformation method overcomes the disadvantages of the conventional in vitro *Agrobacterium*-mediated transformation method. The latter requires sterile condition, that is time consuming and causes somatic mutation or somaclonal variation in plant cells during *in vitro* culture, and some plants are recalcitrant to regeneration. In contrast, *in planta* transformation involves no *in vitro* culture of plants cells or tissue, which is its greatest advantage.

Floral dip transformation

Clough and Bent [68] modified the *Agrobacterium* vacuum infiltration method to transform *Arabidopsis thaliana*. This process was eliminated in favor of simple dipping of developing floral tissues into a solution containing *Agrobacterium tumefaciens*, 5% sucrose and 500 μlL^{-1} of surfactant Silwet L-77. Sucrose and surfactant were critical to the success of the floral dip method. Plants inoculated when numerous immature floral buds and few siliques were produced transformed progeny at the highest rate. Plant tissue culture media, the hormone BAP and pH adjustment were unnecessary, and *Agrobacterium* could be applied to plants at a range of cell densities. Repeated application of *Agrobacterium* improved transformation rates and overall yield of transformants approximately two fold. Covering plants for 1 day to retain humidity after inoculation also raised transformation rates two fold.

Desfeux et al. [69] also investigated the mechanisms that underlie the floral-dip method for *Agrobacterium*-mediated transformation in *Arabidopsis* to facilitate its usage in other plant species.In manual outcrossing experiments, application of *Agrobacterium tumefaciens* to pollen donor plants did not produce any transformed progeny, whereas application of *Agrobacterium* to pollen recipient plants yielded transformants at a rate of 0.48%. Their results suggested that ovules were the site of productive transformation in the floral-dip method, and further suggested that *Agrobacterium* must be delivered to the interior of the developing gynoecium prior to locule closure.

Rod-in et al. [70] reported *Agrobacterium-mediated* transformation method for rice carrying the *gusA* gene to infect rice spikelets via the floral-dip method. The tip-cut spikelets of the rice inflorescence stage 51 (beginning of panicle emergence: tip of inflorescence emerged from sheath) were dipped in the Agrobacterium and co-cultivated at 25 °C for 3 d. Floral dip approach transform primarily anthers and to a small extent the ovary. The highest transformation efficiency in case of anther was 89.16% whereas in case of ovary 7.23% Their results suggest that floral-dip transformation is a simple potential tool for production of transgenic rice with no requirement of tissue culture.

Naseri et al. [71] demonstrated the inoculation of *A. tumefaciens* into embryonic apical meristem of the soaked seeds, a region on the seed surface where a shoot would later emerge and was pierced twice up to depth of about 1 to 1.5 mm with a needle (0.70 mm) dipped in the *A. tumefaciens* inoculums. The inoculated seeds were then placed on filter papers on wet per liter in flasks covered with aluminum foil and incubated at 23°C in dark for nine days that 70 to 75% of inoculated seeds are germinated to seedlings. PCR results showed that 24% plants inoculated with *Agrobacterium* integrated transgene.

Critical parameters: During the *agrobacterium* infection, OD_{600} of the bacterial suspension must be 1.0 for higher transformation.

Source of Explants

Tissue culture which involves manipulating the totipotent nature of plant cells is one of the necessary technology for production of transgenic plants. For genetic transformation in different experiments, many types of explants has been used by researchers likewise mature seeds, mature zygotic embryos, immature embryos, mature embryos, calli, inflorescence, embryogenic apical meristem, spikelet, roots, isolated shoot apices (Tables 1-3).

Vectors and Markers in Gene Transfer

In plant research, binary vectors are standard tools for delivery of a wide range of genes into the cells of higher plants [72] This vector has certain important characters like multiple cloning sites, bacterial origin of replication, unique restriction sites and selectable markers gene cassettes enabling the recognition of untransformed cells. A selectable marker gene encodes a product that allows the transformed cell to survive and grow under conditions that kill or restrict the growth of non-transformed cells. The most used genes in rice are dominant selectable markers that confer resistant to antibiotics or herbicides i.e. *ble* (glycopeptides binding protein), *dhfr* (dihydrofolate reductase), *hpt* (hygromycin phosphotransferase), *nptII* (neomycin phosphotransferase), bar and pat (phosphinothricin acetyltransferase), *csr1-1* (acetolactate synthase), *tms2* (indolaacetic acid hydrolase) (For details see Table 1 [20]. In addition to selectable marker genes, plant researchers also used reporter genes known as screenable marker, a visual marker or a scorable marker,such as *gus A* (ß-glucuronidase, GUS), *luc* (firefly luciferase), *gfp* (green fluorescent protein) (For details see Table 1) [20]. It generates a product that can be detected using a simple and often quantitative assay. It is mainly used for confirming transformation, determining transformation efficiency and monitoring gene or protein activity. It has ability to form fusion genes at the transcriptional level and it can be used to assay the activity of regulatory elements. It also forms translational fusion products, which allows them to be used, to monitor protein localization in the cell or at a whole-plant level. ß-glucuronidase activity can be easily monitored within 1-2 days post co-cultivation in infected plant tissues by enzymatic conversion of colourless X-Gluc substrate to blue precipitates [73] so, it is a powerful assay for detecting transformation events.

Next major aspects of binary vector is the usage of constitutive promoters providing transgene expression in majority of the plant tissues. Studies have been done on rice transformation by using CaMV35s constitutive promoters. By using this promoter Brietler et al. [74] reported 70-80% genetic transformation for *yfp* and *hph* genes. Besides this promoter, there are many others has been used for genetic transformation in rice such as Actin 1, ubi-1, ubiquitin, OSCc1 (Tables 1-3).

Transgenic Analysis

To confirm the real transgenic, efforts should be undertaken with a long term goal rather than just the analysis of the initial transformants. Development of transgenic plants is not only limited to the confinement of laboratory, it should have wider scope of application. So, to test the real transgenic we have to go for different complementary tests like GUS Assay, PCR, Semi-quantitative PCR, Quantitative Real Time PCR, Southern blotting, Western blotting, ELISA, Northern blotting. Most of the studies conducted molecular analysis of the primary transformants, rather than proceeding further. GUS analysis revealed transformation efficiency in wide range (7.23% to 95%) that has been shown by different studies by using different explants in various cultivars of rice [21,75-

Genotype	Explant	Promoter	plasmid	Transgene	Marker gene	Efficiency	Analysis	References
MR219	Mature seeds	35S	pCAMBIA1304	hptII	gusA mgfp5	Regenerability 100%	Southern Analysis GUS staining RT-PCR Flouroscence microscopy Stastical Analysis	Htwe et al. [78]
Swarna Mahsuri	Mature seeds	35S	pCAMBIA1301	Cry1Ac hpt	GUS hygromycin	Regeneration efficiency Swarna (79.23%) Mahsuri(86.07%)	GUS assay Southern blot and PCR Analysis	Pravin et al. [79]
PAU201	Mature seeds	35S	pWRG1515	GUS	GUS	32-39%	GUS Assay	Wani et al. [77]
Rice cv. MR81 Taipei 309	Mature seeds	35S	pRQ6	hph	GUS	>95%	GUS Assay	Rahman et al. [76]
BMS (black mexican sweet) Maize	Mature embryo	35S	pRESQ70	HPH	GUS		PCR and southern analysis	Sivamani et al. [81]
L1345 L3	Immature embryos	35S	Pcambia3301	PAT	GUS	L1345 (20.23-49.38%) L3 (25.19-69.02%)	GUS Analysis	Petrillo et al. [82]
Xiushui04 Jia59	Immature embryo	35S	pCB1	bar			Southern Analysis	Zhao et al. [83]
Rice cv. Chainat 1	Mature seeds	35S	PCAMBIA 1305.1	Chitinase	GUS Hygromycin resistance	100% at 9cm distance from stopping screen to callus and 35.5%at 12 cm distance	PCR Analysis	Maneewan et al. [84]
Rasi Taipei309	Mature seeds	35S	PUCGUS pHX4	hph	GUS	Rasi (15.5-24.2%) Taipei309 (33.3-37.55)	GUS assay	Ramesh et al. [86]
Taipei309 Nippanbare	Mature seeds embryos	35S	pYEP p1LTAB227	Yfp hph		70-80%	Southern blot analysis	Breitler et al. [74]
Eyi105 Ewan 5	Mature seeds	35S	pJIMB15 pRSSGNA1 pAHXA21	hpt gusA Xa21 gna	Hygromycin	Eyi105 (16%) Ewan 5 (18%)	PCR analysis Dot and southern blot analysis RT-PCR Analysis Genetic Analysis	Tang et al. [88]
IR64 IR72 Minghui 63 BG90-2	seeds	35S	pC822 pHX4	Xa21 hph	Hygromycin	IR64(91%) IR72(100%) Minghui 63(54%) BG90-2 (100%)	Southern blot	Zhang et al. [54]
Jingyin 119 Zhongbai 4	Immature embroys	35S Act1	pCB1	Cecropin Bgene	bar		PCR analysis Dot and southern blot analysis Northern blot analysis	Danian et al. [89]
Taipei 309 77125 Tetep TN1 8706	Immature emryo Callus	Actin1 35S	pAct1D pNG3 PMON410	hph	GUS	Taipei 309 (25.6%) 8706 (31.0-42.3%)	GUS assay	Li et al. [21]

Table 2: Trends in rice (*Oryza sativa* L.) transformation through particle bombardment.

77]. PCR analysis showed various range of transformation efficiency i.e. 16%-100% [48-53]. Molecular analysis were carried out by most of the workers to check the integration pattern and copy number by southern blot analysis, northern and real time pcr [78,79,81-84].

Future Prospects

As we look to the future of rice biotechnology, we have many transformation technique but till now no transgenic line of rice has been released successfully. Near about 100 genotypes have been used by different groups for transformation (Tables 1-3). Among them, Taipei 309 (*Japonica* rice) and Basmati (*Indica* rice) has been widely used in *Agrobacterium*-mediated genetic transformation with an efficiency of 31% and 40-75%,respectively.Though some reports has been shown that some of the transgenic line has been released. Gupta et al. [85] reported that Huaghi-1 Rice for insect resistance has been released by Huazhong Agricultural University, China. SO, we have to focus on particular assay of that transgene which has been transferred along with series of confirmatory test. Ultimately we need high yield so, our main objective should be to check our transgene is working or not, which may not affect the yield. As for example, if we transfer salinity tolerance gene, we must go for salinity screening technique, in which transgenic line must show higher tolerance level in respect to wild. In most of the research this assay are lacking. So, to check or confirm real transgenic we must go for particular assay.

Conclusion

In the present scenario, population growth is increasing day by day along with demand for food supply. Genetic transformation must be implemented to bridge the gap between production and human need. Whatever achievement has been done so far in transgenic, is not sufficient to fill the demand. The full realization of the plant biotechnology revolution depends on successful and innovative

Genotype	Explants	Promoters	Strain	Plasmid	Transgene	Marker gene	Efficiency	Analysis	References
RD41	Rice spikelets	35S	AGL1	pCAMBIA1304	hptII	gusA gfp	Anther-89.16% Ovary- 7.23%	Histochemical Gus assay	Rodin et al. [47]
Zhong Zuo321 Nippanbare IR64	Antepenultimate emerged leaf of plantlets at tillering stage	Ubi-1 CsVMV	EHA101 AGL1 LBA4404 GV2200 GV3101	pC5300 PB10S738	UidA Sc4A	GUS	-	Real time PCR Analysis	Andrieu et al. [87]
Oryza sativa	Seeds	35S	EHA101	pAJ21	TLPD34		24%	PCR Analysis	Naseri et al. [71]
Koshihikari	Embryonic apical meristem	35S	M-21 LBA4404	P1G121Hm	Hpr nptII	GUS	40-43%	PCR	Supartana et al. [90]
Taipei 309	Infloroscence	35S	LBA4404	PJD4	Bialaphos resistance gene	GUS	-	GenomeDNA blot analysis PCR analysis	Dong et al. [91]

Table 3: Trends in rice (*Oryza sativa* L.) transformation through in-planta transformation.

research, as well as on favorable regulatory guidelines and public acceptance. Thus, all the strategies discussed in the present review will definitely contribute to biotechnological breeding programs of rice for its improvement.

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