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Biotehnology

Agrobacterium Mediated Transformation in Rice: A Novel Approach to Produce Agronomically Superior Transgenics

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Abstract

Rice the most important staple grain with second highest worldwide production has offered a great potential for biotechnological advances, categorized broadly in areas like transgenics, structural and functional genomics and marker-assisted breeding to provide with vital breakthroughs to achieve improvements in both quality and quantity in a sustainable manner. The ever increasing demand, increasing anthropogenic activities has posed a challenge to boost the production of rice. To cope of with this alarming situation, the advent of genetic engineering has been utilized to remove natural barriers through gene transfer/exchange. Since then, sequences from various sources like bacteria, viruses and eukaryotic systems were used to develop transgenic crop varieties. Gene transfer techniques like direct DNA transfer through particle bombardment, polyethylene glycol or electroporation and indirectly mediated by Agrobacterium are increasingly applied. Unfortunately, DNA delivered by direct methods is often integrated into the genome as rearranged or multiple copies, presumably as a result of which the expression pattern of the gene of interest is often aberrant or unstable. In contrast to this, Agrobacterium mediated transformation system offers higher transformation efficiency with discrete, unrearranged segments of DNA being inserted at fairly low copy number. Achievements, to date, through Agrobacterium mediated transfer have surpassed all previous expectations and with the development of this successful technique for genetic improvement the future is even more promising.

Highlights

- Agrobacterium mediated transformation system offers better transformation efficiency than indirect transformation methods.
- Agrobacterium mediated transformation offers discrete, unrearranged segments of DNA being inserted at fairly low copy number.
- This method allows the genetic improvement of diverse varieties of rice as well as study of many aspects of molecular biology.

Keywords: Agrobacterium tumefaciens, genetic transformations, rice, transgenic

Rice Biotechnology

Rice is one of the most important cash crop in the world that feeds more than two billion people all around with more than 90% being consumed by the Asian (Datta, 2004). Rice member of family Gramineae and genus Oryza has twenty one wild species and two cultivated species, of which the cultivated species are diploid 2n = 24 and have the genome AA, while wild species are either having 2n= 24 or 2n= 48 chromosomes representing AA, BB, CC, BBCC, CCDD, EE, FF, GG and HHJJ genomes (Khush, 1997). There is adequate amount of genetic variability present in the material (Singh *et al.,* 2013).

The wild type species provide a pool of genes which are resistance to various diseases and insects however, gene transfer between wild and cultivated species is difficult owing to limited recombination and low crossability of the chromosomes. But with the advent of genetic engineering, it became possible to introduce varietal improvements through genes transfer from within as well as outside the gene pool (Grover and Minhas, 2000). Now, rice has become a model system for cereal genomic research due to its small genomic size (4.2 X 108bp) and versatile nature to adapt to different geographic locations, environments and soil types (Ou, 1985 and Arumuganathan and Earle, 1991). Owing to all these factors and ever increasing demand of the crop, it becomes imperative to introduce new improved varieties with higher yields, multiple resistances to pests and diseases and tolerance to various stress conditions.

Gene transformation

The most commonly used gene transfer methods in plants are either mechanical or biological. Mechanical methods include protoplast mediated transformation, microprojectile bombardment of cells and tissues,

Table 1. Major transgenic research conducted on rice in Indian laboratories (Sharma *et al.*, 2003).

Transgene(s) inserted	Aim of research	Laboratory/ Insitute
S-adenosylmethionine Decarboxylase	To generate plants tolerant to stress	Bose Institute, Kolkata
Bar	To generate herbicide-tolerant plants	Centre for Cellular and Molecular Biology, Hyderabad
Bt cry1A(b) Xa21	To develop plants resistant to lepidopteran pests, bacterial blight/disease	Central Rice Research Institute, Cuttack
Pyruvate decarboxylase and alcohol dehydrogenase	To generate plants tolerant to flooding	Delhi University, South Campus, New Delhi
codA, cor47,	Resistance against biotic and abiotic Stresses	Delhi University, South Campus, New Delhi
Bt cry1A(b), chitinase	To generate plants resistant to lepidopteran Pests	Indian Agricultural Research Institute, New Delhi
Bt. cry1A(b)	To generate plants resistant to yellow stem borer	IARI sub-station, Shillong
Gm2	To generate plants resistant to gall Midge	International Centre for Genetic Engineering and Biotechnology, New Delhi
Chitinase, b-1, 3-glucanase and osmotin genes	To develop plants resistant to fungal Infection	Madurai Kamaraj University, Madurai
Cry1A(b) gene	To generate plants resistant to lepidopteran pests, bacterial and fungal Diseases	Narendra Dev University of Agriculture, Faizabad
Cry1Ab, Cry1Ac	For resistance against yellow stem Borer	Punjab Agricultural University, Ludhiana
GNA gene	To generate plants resistant to pests gall midge	Tamil Nadu Agricultural University, Coimbatore
Cry1A(b), cry9C and bar genes	To develop plants resistant to lepidopteran pests and herbicide tolerance	Hybrid Rice International, Gurgaon
Cry1A(c), Xa21 and GNA genes	To generate plants resistant to lepidopteran pests, bacterial blight and sucking pests	MAHYCO, Mumbai
Bacterial blight resistance conferring Xa-21 gene	To generate plants resistant to bacterial blight	MAHYCO Research Foundation, Hyderabad

electroporation and polyethylene glycol (PEG) (Gould 1996, Christou *et al.,* 1997, Toriyama *et al.,* 1985) while biological methods evolved from Agrobacterium-mediated gene transfer. A vast degree of transgenic research employing all these methods is undergoing in various Indian laboratories (Table 1).

However, use of mechanical methods of gene transfer has not been as successful in producing transgenics as compared to biological methods owing to significant drawbacks. Biological method involves Agrobacterium tumefaciens, a gram negative soil bacterium that has evolved a unique gene transfer mechanism to infect, transform and parasitize plants. Bacteria can penetrate wounded cell sites and actively integrates the T-DNA stably into plant chromosomes (Ratnayaka, 1999). The Agrobacterium mediated method also facilitates transfer of large DNA segments (more than ten genes) without any rearrangement, which in turn provides an opportunity to study cumulative, interactive effects on complex polygenic traits (Hamilton *et al.,* 1996). Moreover, the process is inexpensive and it does not require the use of protoplasts (Komari *et al.,* 1996, Park, 1996).

More than a decade have elapsed, since the advent of biological system of gene transfer mediated through Agrobacterium has revolutionized the field of rice research, but method remained concealed because the scientific community still considered cereals to be refractory to Agrobacterium (Vasil 1994, Vijayachandra *et al.,* 1995). However, in 1993 Chan and co-workers convinced the scientific society by successfully culturing immature embryos of japonica rice plant with Agrobacterium to obtain transgenic. The inheritance of transformed DNA to the progeny was ascertained by Southern hybridization, although the progeny of only one plant was analyzed. Hiei *et al.,* 1994 used different cultivars of japonica rice, namely, Oryza sativa L. Tsukinohikari, Asanohikari and Koshihikari, for transformation by Agrobacterium. Calli derived from scutella, which proved to be the most suitable material for infection was co-cultivated with EHA101 (piG121 Hm, pTOK233) and LBA4404 (pTOK233, piG121 Hm) for 3 days in the presence of 100 µM acetosyringone. The highest transformation frequency (23%) for hygromycin-resistant cells was observed. A successful integration and expression of the reporter gene, β-glucuronidase (GUS) and plant selectable marker gene, hygromycin phosphotransferase (Hpt) in R1 and R2 transgenic plants was achieved.

Vijayachandra *et al.,* (1995) used scutellum derived callus for Agrobacterium- mediated transformation in rice and suggested that pretreatment of A. tumefaciens with vir inducing compounds like acetosyringone and potato suspension, before co-cultivation enhances transformation efficiency. Scutellum-derived calli was considered to be the most desirable material of choice for efficient transformation in rice (Hiei *et al.,* 1994). Aldemita and Hodges, (1996) used immature embryos and shoot apices of japonica and indica rice varieties for stable integration and inheritance of GUS reporter gene via Agrobacterium mediated transformation. Dong *et al.,* 1996 showed production of fertile; stably Agrobacterium mediated transformed plants from japonica cultivar Taipei 309 and japonica cultivar Gulfmont. Similarly, Park *et al.,* 1996 described the transfer of gene coding for glufosinate-ammonium (PPT) resistance into japonica rice by using Agrobacterium and confirmed the inheritance of the transferred genes to the progeny by Southern blot analysis. Hiei and Komari, 1997 analyzed the inheritance of transgenes to R4 generations successfully for eighteen transgenic plants that have been produced by the Agrobacterium-mediated method. Latter, a number of workers used the same procedure for stable genetic transformation in indica rice varieties (Rashid *et al.,* 1996, Khanna and Raina, 1999, Mohanty *et al.,* 1999).

Hiei *et al.,* (1997) also highlighted the importance of Agrobacterium mediated transformation in rice to allow diverse genetic improvements. The study of transformation of rice suggested that numerous factors including genotype of plants, types and ages of tissues inoculated, kind of vectors, strains of Agrobacterium and various conditions of tissue culture are of immense importance. Keeping in view all the factors controlling integration, inheritance and expression of transgenes, Chen *et al.,* (1998) successfully developed one hundred and twenty-five independent transgenic rice plants after co-bombarding embryogenic tissues with a mixture of 14 different pUC- based plasmids. Khanna and Raina, 1999 used binary and super binary vector based system for Agrobacterium-mediated transformation in indica rice cultivars. A high efficiency rate of 1,000 stable transformants from 150 seed-derived calli in japonica rice

was obtained by Terada *et al.,* 2004. Hiei *et al.,* (2008) also provided a comprehensive and highly efficient Agrobacterium tumefaciens mediated transformation protocol for a wide range of rice genotypes like japonica and indica. Transformed cells selected on the basis of hygromycin resistance produced between 10 and 18 independent transgenic plants for japonica varieties and between 5 and 13 plants for indica varieties. Sahoo and Tuteja, 2012 developed a simple, rapid and improved genetic transformation protocol for the indica rice cultivar IR64 using Agrobacterium-mediated genetic transformation. Scutellum explants immersed in Agrobacterium suspension showed maximum transformation efficiency (12%) with integration of the transgenes in T1 transgenic plants confirmed by polymerase chain reaction and Southern blot analyses.

Table 2. *Agrobacterium* mediated transformations in rice

Variety/cultivar	Explant	Configuration of the plasmid construct	Reference
Nipponbare, Fujisaka 5 (japonicas)	Mature embryos	pMas/35S:gus	Raineri *et al.,* 1990
8 indicas, 7 japonicas, 6 African rice varities	Leaf, root, seed	p35:gus/intron* pMas/35S*gus pMas/35S*:gus/intron	Li *et al.,* 1992
IR 64 (indica), Lemont	Shoot segment	p35:gus/nos* p35S:gus.ocs* p35S:gus/intron.ocs*	Li *et al.,* 1992
Tainung 629japonica)	Immature embryos	pNos:nptII:paaAmy8*:uidA	Chan *et al.,* 1993
Tsukinohikari, Asanohikari, Koshihikari (japonicas)	Shoot segments, root segments, root derived calli, scutella derived calli, cell suspensions, immature embryos	pNos:nptII:p35S: intron:gus:p35S:hpt	Hiei *et al.,* 1994
Co43(indica)	coleoptile, scutellar callus, leaf blade, leaf base, root	virE-lacZ, vir13-lacZ* extra copies of virG,C,D,E*	Vijaychandra *et al.,* 1995
Nortai, Radon (Japonicas)	Immature embryos	pMas/35S:gus pNos: nptf p35S: intron.- gus: p35S: hpt	Aldemita & Hodges 1996
Taipei 309 (japonica), Gulfmont, Jefferson (javanica)	Scutellar calli	pNos:nptII, p35S:intron:gus, p35S:hpt	Dong *et al.,* 1996
Maybelle (japonica)	Shoot meristems	p35Sbar pAct1:acf1:bar:pNos:nptII	Park *et al.,* 1996
B370 (indica)	Scutellar calli	pNos:nptII, p35S:intron:gus, p35Shpt	Rashid *et al.,* 1996
Binnatoa (indica)	Scutellar calli	pNos: nptII, p35:intron:gus, p35S:hpt	Rasul *et al.,* 1997
Nipponbare, Kitaake (japonicas)	Seed callus	pNos:nptII, p35S:intron:gus, p35S:hpt:Nos:hpt:pUbi:bar	Toki, 1997
Taipei 309(japonica)	Scutellar callus	p35S:hpt:nos, p35S:hpt.tm1* p35S:hpt-cat-hpt p35S:hpt-haem-hpt pUbi:ubi.hpt	Wang *et al.,* 1997

Nipponbare (japonica)	Scutellar callus	pNos:nptII::p35S:sodCc2*, coda::p35S:hpt pNos:nptII::p35S:sodCc2*:TP:coda::p35S:hpt	Sakamoto *et al.,* 1998
Yamahoushi (japonica)	Seed callus	Kmr*:pUbi:gpat*:Hygr	Yokoi *et al.,* 1998
Nipponbare (japonicas)	Seed callus	pNos:nptII:pepc:p35S:hpt	Ku *et al.,* 1999
DS20,OMCS96, OMCS97, IR72 and IR64 (indicas)	Seed callus	pSBbarB-UbiCre: pSB35L-Hyg-L-Gus.	Hoa *et al.,* 1999
Chaitanya, Phalguna and Swarna, besides three parents, viz. IR58025A (CMS line), IR58025B (maintainer line) and Vajram (restorer)	Seed Callus	pTOK 233, pSB111-ubi-cry1Ab/cry1Ac-35S-bar and pSB111-RSs-gna-35S-bar	Ramesh *et al.,* 2004
Nipponbare (Japonica rice)	Scutellum tissue	pCAMBIA1390-sGFP	Toki *et al.,* 2006
Bg 250(indica rice)	Seed callus	pCAMBIA 1303: GUS	Ratnayake and Hettiarachchi, 2010
Sented indica, Tararori basmati, IR 70485-15-3-2	Seed callus	Am-SOD: CaMV 35S: Nos:pCAMBIA 1301	Sarangi *et al.,* 2011
IR-64, Lalat, and IET-4786 (indicas)	Seed callus	pIG121-Hm	Shri *et al.,* 2012

Genes of agronomic importance transferred in rice through Agrobacterium

A number of genetic engineering approaches have been attempted to improve stress tolerance in plants (Shinozaki *et al.,* 2003). A number of attempts have been made by scientists to combat biotic and abiotic problems in plants. Some of the commonly existing strains in rice include:

Salt tolerance

Salinity is one of the major factors that limits the production and distribution of cereal crops. Thus, engineering of crop plants against salt tolerance is a major challenge in gene technology (Boyer, 1982). In an attempt to induce salt tolerance in rice- a salt sensitive cereal crop which cannot produce glycinebetaine as a consequence of the apparent absence of the activities of the two enzymes, Sakamoto and Alia, 1998 obtained Agrobacterium mediated transgenic rice (Oryza sativa L.) with the ability to synthesize glycinebetaine by introducing the codA gene for choline oxidase from the soil bacterium Arthrobacter globiformis. This transgenic variety accumulated glycinebetaine and acquired enhanced tolerance to salt and cold.

Similar effort was made by Islam *et al.,* 2009 wherein in order to improve salinity tolerance in rice, Agrobacterium mediated transformation and constitutive expression of PgNHX1 from Pennisetum glaucum L in Bangladeshi rice Binnatoa was studied. Four T0 tolerant progenies with more tolerance than untransformed were observed and named PgV-186, PgV-241, PgV-242 and PgV-247. Best performing PgV-247 during screening test selected when analyzed through southern blot confirmed single site integration. In another study to induce salt tolerance, Abdullah-Al-Emran *et al.,* 2010 cloned SNAC1 gene from the salt tolerant Pokali variety into pETNR/D-TOPO vector and subsequently transferred it into the destination vector (pH7WG2) by LR recombination for Agrobacterium mediated transformation of the Bangladeshi indica rice landrace Binnatoa.

Table 3. *Agrobacterium* mediated transgenic lines in rice for drought tolerance

Transgenes	Source organism	Transformation methods	Trait improved	Reference
SNAC 1.	Oryza sativa L	Agrobacterium	Transgenic plants showed improved tolerance to drought conditions.	Hu *et al.,* 2006
Os LEA-3–1	Oryza sativa L.	Agrobacterium	Transgenic plants showed increased growth under drought conditions	Xiao *et al.,* 2007
HvCBF4	Hordeum vulgare L.	Agrobacterium	Improved drought and salinity tolerance	Oh *et al.,* 2007
Tomato ethylene response factor (ERF) protein TSRF1	Tomato (Lycopersicon esculentum L.)	Agrobacterium	TSRF1 improved the osmotic and drought tolerance of rice seedlings without growth retardation	Quan *et al.,* 2010
Tomato ethylene response factor (ERF) protein JERF1	Tomato (Lycopersicon esculentum L.)	Agrobacterium	Over expression of JERF1 significantly enhanced drought tolerance of transgenic rice	Zhang *et al.,* 2010
OsDREB2A gene with stress-inducible promoter (4ABRC)	Rice (Oryza sativa L.)	Agrobacterium	Over expression of OsDREB2A significantly enhanced drought and salt tolerance of transgenic rice	Cui *et al.,* 2011
Rice OsSDIR1 gene	Rice (Oryza sativa L.)	Agrobacterium	Over expression of OsSDIR1 gene significantly enhanced drought and salt tolerance	Gao *et al.,* 2011
Sorghum SbDREB gene with stress induced promoter CaMV35S or rd29A	Sorghum bicolorL	Agrobacterium	Over expression of SbDREB2 significantly enhanced drought tolerance and yield improvement in transgenic rice	Bihani *et al.,* 2011
Rice OsSDIR1 gene	Rice (Oryza sativa L.)	Agrobacterium	Over expression of OsSDIR1 gene significantly enhanced drought and salt tolerance	Gao *et al.,* 2011
OsDREB2A gene with stress-inducible promoter rd29A	Rice (Oryza sativa L.)	Agrobacterium	Over expression of OsDREB2A significantly enhanced drought and salt tolerance of transgenic rice	Mallikarjuna *et al.,* 2011

Diseases resistance

Resistance against viral, bacterial and fungal infections is of major concern in rice because insects attack all parts of the plant and exert physiological damage to the plant thereby attracting various pest species such as stem borers, leaf folders, plant hoppers and gall midges (Maclean *et al.,* 2002). A number of researchers have engineered effective and safe ways of developing resistance against these pests. One such method involves Agrobacterium strains for genetic manipulations. Lin *et al.,* 1995 reported the production of transgenic rice expressing Chl11, which showed protection against sheath blight pathogen (fungus) Rhizoctonia solani. Similarly, Sridevi *et al.,* 2003 and Datta *et al.,* 2001 has also reported significant levels of resistance against the fungal pathogen using same gene. Chang *et al.,* 1998 regenerated over 2,600 transgenic Agrobacterium-transformed rice plants expressing synthetic cryIA(b) and cryIA(c) genes which were highly toxic to striped stem borer and yellow stem borer thus offering a potential for effective insect resistance in transgenic rice plants.

A gene pyramiding approach used by Datta *et al.,* 2002 involved Xa21, for resistance to bacterial blight, cry genes for insect resistance and RC7 for sheath blight resistance. Agrobacterium mediated transformation in rice plants expressing synthetic cryIA(b) and cryIA(c) genes highly toxic to two major rice insect pests- striped stem borer (Chilo suppressalis) and yellow stem borer (Scirpophaga incertulas) was successfully achieved by Cheng *et al.,* 1998. Hirose *et al.,* 2005 used Agrobacterium-mediated transformation of human gene for CYP2B6 (a cytochrome P450 monooxygenase that inactivates xenobiotic chemicals) into Oryza sativa cv. Nipponbare. R1 seeds of transgenic rice plants expressing CYP2B6 (CYP2B6

rice) showed a high tolerance to 5 µM metolachlor, a preemergence herbicide that is degraded by CYP2B6 and were able to grow in the presence of 13 out of 17 herbicides.

Drought tolerance

Drought posed biggest challenge to agricultural scientist as it can occur at any point during crop production with the uncertainty in drought timing, intensity and duration affecting a large array of physiological, biochemical, and molecular processes.

Wang *et al.,* 2005 introduced MnSOD an anti-oxidant enzyme from pea (Pisum sativum) under the control of an oxidative stress-inducible SWPA2 promoter into the chloroplasts of rice (Oryza sativa) via Agrobacterium-mediated transformation to develop drought-tolerant rice plants.

Conclusion

From the present manuscript it can be concluded that ample work is done on crop improvement through various genetic transformations. However, there is still need for carrying out research on creating more superior transgenic rice plants that can grow in compromised environments and decreased arable land with higher yields.

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