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Agrobacterium-Mediated Transformation of Rice: Constraints and Possible Solutions

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Abstract: Genetic transformation of rice (*Oryza sativa* L.) by introducing beneficial traits is now a central research instrument in plant physiology and a practical tool for plant improvement. Many approaches are verified for stable introduction of foreign genes into the plant genome. The review examined the different constraints that limit the success of rice genetic transformation via *Agrobacterium*-mediated approach and suggested possible solutions. Explant identification, gene transfer technique and construct to tailor the integration, transgene expression without collateral to genetic damage and transformant selection are among the technical challenges affecting the rice transformation. Despite the contests, *Agrobacterium*-mediated transformation system has been a better option for producing transgenic rice varieties because of its exact T-DNA processing and simple integration of low copy-number transgene. This information is necessary for improving the transformation system for recalcitrant rice varieties.

Key words: rice; *Agrobacterium*-mediated transformation; tissue culture; gene transfer; T-DNA integration

Genetic transformation occurs naturally in bacteria (Lorenz and Wackernagel, 1994; Laurenceau et al, 2013), whereas in other organisms such as plants, it is achieved by artificial mechanisms (Koziel et al, 1993; Tzfira and Citovsky, 2006). Horsch et al (1984) proved the capacity to introduce a diverse foreign gene(s) *in vitro* into the plant organism. The successful regenerations of transgenic fruits (Yao et al, 1995; Mulwa et al, 2015), vegetables (Zhang and Blumwald, 2001), medicinal and ornamental geophyte (Koetle et al, 2017) and crops such as rice (Zhang et al, 1988; Hiei et al, 1994; Saika and Toki, 2010; Manimaran et al, 2013; Fook et al, 2015) have been reported. Therefore, floral transformation is now a core research and a practical tool for cultivar improvement. There are well-established systems for stable and effective introduction of novel genes into the genomes of plant species (Ziemienowicz, 2014). The pioneered applied method to generate fertile transgenic plants (dicots and monocots) includes electroporation (Ou-Lee et al, 1986; Toriyama et al, 1988), polyethylene glycol (PEG) mediated transformation (Datta et al, 1990) and an electric discharge particle acceleration device (Christou et al, 1991).

Conservatively, monocots are only transformed using the above-aforementioned direct gene transfer techniques (Lörz et al, 1985), while *Agrobacterium*-mediated transformation system has been used for the transformation of dicots. Monocots are not the natural hosts of *Agrobacterium* species, and thus transformation of monocot is extremely tedious (Sood et al, 2011). The initial monocotyledons used to transform using the *Agrobacterium*-mediated approach comprised rice, wheat and maize (Sood et al, 2011). In 1994, rice was strongly believed to be amenable to *Agrobacterium*-transformation (Hiei et al, 1994). Progressively, due to

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the advent of molecular techniques and understanding of plant physiology, the incoming of super-binary vector construct and bacterial strains, the mediated *Agrobacterium* system is accepted and numerous monocots are transformed (Cheng et al, 2004). However, transformation technology of rice is often hampered by lack of specific efficient method, defined explant(s), regeneration conditions as well as low cell competence.

One of the technical challenges fronting the transformation toward producing stable transgenic rice is how to produce high proportion of transformants with a predictable transgene expression without collateral to genetic damage. Despite the contests, *Agrobacterium* transformation system has been a better option by troubleshooting the parameters that govern efficient transgene delivery and integration into the plant genome (Hiei et al, 1994; Izawa and Shimamoto, 1996; Gelvin, 2000; Xu et al, 2017). In this review, key problems, constraints impeding the research and development of rice transformation system, and key issues to be resolved in the practical application with more emphasis on *Agrobacterium*-mediated transformation as the superlative method for rice genetic transformation are discussed. Such information is necessary for the biotechnological improvement of rice species.

Importance of rice production

The over-dependent on rice as staple diet remains unshakable over decades. Therefore, increasing rice yield remains one of the most important goals in plant science research, such as agriculture and crop production (Xue et al, 2008), especially in Asia and Africa nations (Karthikeyan et al, 2009). As reported by Karthikeyan et al (2009) and Buttimer et al (2017), there is an increase in the demand for rice due to over-growing of world population and it is essential to upsurge a domestic production for food security purpose. Hence, biotechnology intervention through tissue culture technology and genetic transformation is the solution (Azhakanandam et al, 2015).

Importance of rice transformation

With the advent of molecular techniques, plant transformation has become possible. The capacity to manipulate genetic material by introducing and expressing a specific novel-foreign gene(s) in plants provides a powerful new experimental tool, allowing direct testing of hypotheses in plant physiology that have become exceedingly difficult to resolve using conventional breeding or biochemical tests (Birch, 1997). The exciting examples include the molecular genetic analysis of cellular signals controlling the sexual reproduction in rice (Komiya et al, 2008, 2009), and molecular mechanism of the roles of specific proteins, hormones and enzymes in plant metabolism and development (Kang and Turano, 2003; Li et al, 2006; Lee and An, 2015).

The transformation approach might generate useful plants with special phenotypes that is unachievable by conventional breeding approach, rectify faults and improve physiological and agronomical traits in some cultivars more professionally (Birch, 1997). Interestingly, certain prospects have been met with the generation of commercial transgenic plant lines expressing transgenes conferring resistance to insects, pathogens, environmental stress and herbicides, or increasing grain yield and weight (Table 1). Another newly exciting event is the recent constructions of transgenic lines with useful product for pharmaceuticals (Arntzen, 2015; Ma et al, 2015; Sack et al, 2015) and biodegradable plastic (Snell and Peoples, 2009; Wang et al, 2010; Hempel et al, 2011). Moreover, the extent to which additional commercial, practical or agronomically improved rice can be met totally depends on the efficiency of transformation protocol that can produce lines without any genetic damage. Such experiment requires a series of screening of the transformants expressing the desired transgene, as well avoid misleading result from unintended genetic alteration during the process.

Agrobacterium-mediated transformation

The genus *Agrobacterium* has five species which are classified based on their mode of infection as follows: *A. radiobacter* as an 'avirulent' species, *A. rhizogenes* causing hairy root disease, *A. rubi* causing cane gall disease, *A. tumefaciens* causing crown gall/tumour disease and *A. vitis* inducing galls on grape. Perhaps, a further reasonable system of classification has further divided the genus into 'Biovars' based on the species metabolic characteristics and growth mode (Mafakheri et al, 2017). Genus *Agrobacterium* is a Gram-negative, soil pathogenic bacterium which causes the formation of crown tumors near to infection sites in its host plant. The genus *Agrobacterium* possesses the ability to

Table 1. Rice genotypes, medium for explant culture and regeneration, and their improvements via *Agrobacterium*-mediated transformation.

Cultivar	Sub-species	Medium [a]	Target explant	Improvement trait	Reference
Pusa Basmati	*indica*	MS	Embryogenic callus	Development of fertile transgenic rice	Mohanty et al, 1999
Sambha Mahsuri, Cotton Sannalu, Pusa Basmati, Taraori Basmati	*indica*	MS	Embryogenic callus	Abiotic stress (salt) resistant; *SOD* gene	Sarangi et al, 2011 Reddy et al, 2019
IR64	*indica*	MS	Shoot apex	Antibiotic resistant plant	Dey et al, 2012
Cempo Ireng	*indica*	2N6, MS	Embryogenic callus	Early flowering development	Purwestri et al, 2015
MR219	*indica*	MS	Embryogenic callus	Overexpression of stress related gene, *Auxin Binding Protein 57* (*Abp 57*)	Tan et al, 2017
Sambha Mahsuri, Cotton Sannalu	*indica*	N6, MS	Embryogenic callus	Improvement of drought tolerance	Reddy et al, 2018
Tsukinohikari, Asanohikari, Koshihikari	*japonica*	N6	Immature embryo	*Hygromycin* resistant plant	Hiei et al, 1994
Nipponbare	*japonica*	N6	Embryogenic callus	Identification of *HAP2* genes, 11 *HAP3* genes and 7 *HAP5* genes binding to the CCAAT-box	Thirumurugan et al, 2008
Nipponbare	*japonica*	MS	Embryogenic callus	Resistance to drought, salinity and pathogens and increasing photosynthesis potential and tiller number	Alam et al, 2015
Nipponbare	*japonica*	YN	Embryo	Heat tolerance	Liu et al, 2016
Dongjin	*japonica*	YN	Embryo	Increase tolerance to cold stress, *OsCYP19-4* gene	Yoon et al, 2015
Dongjin	*japonica*	2N6	Embryogenic callus	Promote flowering in rice at short-day condition	Han et al, 2015
Taipei 309	*japonica*	MS, N6	Embryogenic callus	Iron vitamin improvement	Lucca et al, 2001
Taipei 309	*japonica*	MS	Embryogenic callus	Early development of rice inflorescence	Prasad et al, 2003
Zhonghua 11	*japonica*	N6	Immature embryo	Floral activator *OsELF3* controlling heading date at long-day condition	Yang et al, 2013
Zhonghua 11	*japonica*	MS	Embryogenic callus	Increase plant height, grain yield and grain weight	Zhu et al, 2017

[a] MS, Murashige and Skoog medium; N6, N6 medium; YN, Yoshida nutrient.

transfer foreign DNA into the plant genome by horizontal gene transfer (Escobar and Dandekar, 2003). *Agrobacterium* genes are required to establish tumorigenesis and to bring about opine biosynthesis. For example, during transformation, the *Agrobacterium* species facilitates the delivery of transferred-DNA (T-DNA) into the host genome, as it possesses stable and efficient mechanism. *Agrobacterium*-plant gene transfer can be categorized into five crucial steps, including induction of *Agrobacterium* virulence system, generation of T-DNA complex, T-DNA transfer to the plant cell nucleus, integration of T-DNA into the plant genome and T-DNA gene expression by transformed plant (Ziemienowicz, 2014).

Agrobacterium-mediated transformation mechanism indicates that the *Agrobacterium* cell transfers its T-DNA into the nuclear genome of the host plant. Normally, the T-DNA resides in the bacterial growth-inducing plasmid [tumor inducing (Ti) or root inducing (Ri)], and later sliced by virulence proteins (Gelvin, 2000; Bourras et al, 2015). The virulence genes (*vir*-genes) are mostly polar in nature that are located around the T-DNA borders of the plasmid. Therefore, the *vir*-genes play a critical role in generating T-strand or T-DNA by the bacterium, T-DNA transfer or formation of T-complex and its transportation into the plant cell via the T-pilus and T-DNA deliverance up to the actual plant genome as well as opines secretion (Gelvin, 2000). *Agrobacterium*-mediated DNA transfer system offers unique advantages over direct gene transfer techniques as follows: ease of the gene transfer, precise foreign gene transfer and simple integration of the DNA sequence with defined ends, a linked transfer of selectable marker along with the gene, low copy number of the transgene, higher frequency of stable transformation, reasonably lower rate of transgene silencing and ability to transfer long stretches of T-DNA (up to 150 kb) (Veluthambi et al, 2003).

As reported by Sood et al (2011), due to the straightforwardness of the *Agrobacterium* Ti plasmid-based vector transformation and precise integration of single copy number of transgene into the plant genome, the technique continues to be the most used and the best method for rice transformation so far, and the bacterium species effectiveness is satisfactory (Manimaran et al, 2013). Previous findings expressed the competence of *A. tumefaciens* towards achieving a successful production of transgenic rice (Endo et al, 2015), therefore, the genus *Agrobacterium* is called 'natural plant genetic engineer' (Chandra, 2012; Hoffmann et al, 2017). However, certain factors such as genotype negatively influence the processes, and

overcoming such constraints is noteworthy in genetic transformation of rice. A complete schematic representation of *Agrobacterium tumefaciens*-mediated transformation of rice is shown in Fig. 1.

Technological constraints

Tissue culture and regeneration constraint

The choice of transformation technique affects various secondary parameters such as tissue culture, transfer of gene, transgene integration and gene expression (Tan et al, 2017). Perhaps, the number of produced transgenic plants are usually the factor to define the functionality of a particular system (Birch, 1997). The most critical steps in the genetic transformation of the plant include identification of viable host tissue or cells, foreign DNA integration into the viable plant nuclear genome and regeneration of transgenic plant (Ziemienowicz, 2014; Rashid et al, 2017). Plant tissue culture technology presents an appropriate system for plant propagation through culture of diverse organs (explant) as means of genetic transformation and transgenic plant regain. Tissue culture is prerequisite for plant transformation as it is employed in almost all current transformation techniques to achieve a practical efficiency of foreign gene introduction and regeneration of transformants (Hellwig et al, 2004). Hence, this resourceful invention system serves as a mechanism for accelerating the genetic improvement via genetic transformation. With this technological approach, great improvements in monocot genetic manipulation including rice are accomplished (Cheng et al, 2004; Ullah et al, 2007). The basic anatomy of such explants has been implicated to govern the response to the transformation infection (Sood et al, 2011). Rice immature embryo, embryogenic callus or shoot apex are usually produced during tissue culture and used as host plant. The responses of explant cells primarily depend on the species genotype which serves as a major factor for determining a successful rice transformation (Sahoo et al, 2011).

Genotype dependence is key issue in rice transformation, as it causes undesired change(s) during culture, for example, somaclonal variation. Due to such constraint, T-DNA fails to target the specific meristematic cells that are competent to dedifferentiate. According to Bhatia et al (2017), differences in dicots and monocots cell wall chemistry are thought to determine the success in *Agro*-transgene infection. The dicot cell wall constitutes the glucuronoarabinoxylans, β-linked glucose residues with linking chains of β-*D*-xyloglucans and linear chains of β-*D*-xylose, which are characterized to interlock the polysaccharides in grass species (Grabber, 2005; Vogel, 2008). As an alternative of hydroxyproline-rich extensions that normally accumulate in the cell walls, threonine-rich proteins with sequences evocative of extension are observed during cellular differentiation in monocots (Xing et al, 2009; Sood et al, 2011). The monocotyledonous meristematic cells affect the *Agrobacterium* pathogenicity ability which lower the function(s) of its *vir*-genes (Xing et al, 2019). For that, the cells would fail to exude the inducing compounds of such genes into the plant cells, losing the capacity to dedifferentiate at early stage of development (Lowe

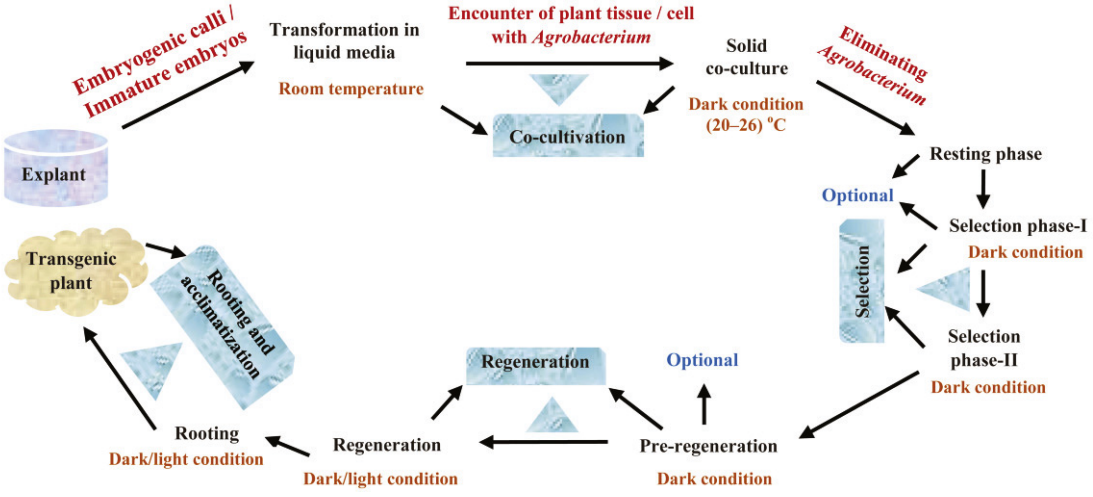

Fig. 1. A complete schematic representation of *Agrobacterium*-mediated transformation and recovery of transgenic rice adapted from Shrawat and Lörz (2006).

et al, 2016; Mafakheri et al, 2017).

Various explants have been used in rice transformation, namely embryogenic callus, immature embryo and shoot apex. The callus potential to regeneration after the genetic transformation is cumbersome and variety-dependent with limiting efficient. Whereas, immature embryos are available only at certain period and are difficult to handle (Dey et al, 2012). Therefore, the use of rice shoot apex for transformation and transgenic recovery via *Agrobacterium*-mediated transformation is a possible solution. The merits with shoot apex transformation include being genotype-independence, direct regeneration of transformants due to developmental plasticity of the plant part, maintaining cultivar integrity and ease of handling (Fook et al, 2015; Clement et al, 2016).

With respect to the above monocot inability to *Agrobacterium* infection, it is generally believed that the success of transformation depends on the explant physiological status (Birch, 1997; Danilova et al, 2006; Lacroix et al, 2011; Koetle et al, 2015; Ithape et al, 2017; Rashid et al, 2017). Many rice cultivars are unable to dedifferentiate and produce tumor after transformation. To some extent, few varieties produce callus in high cell proliferation index (Pathi et al, 2013; Din et al, 2016), but, determination of rice genotype with high embryogenecity and its specific explant for transformation are fiddly, which is another factor causing the poor transformation potential in rice (Sood et al, 2011). Remarkably, such defect has been revitalized by extensive optimization of the callus induction and transformation parameters (Cheng et al, 2004; Saika and Toki, 2010; Mann et al, 2012; Shi et al, 2017). In contrast, embryogenic callus induction from *indica* rice varieties fails to respond to the treatments such as plant growth regulators supplementation and media type, displaying less transformation efficiencies as recorded (Uzé et al, 2000; Tie et al, 2012).

Media for tissue culture and regeneration affect the development of transgenic rice (Table 1), and their supplements can improve the transgenic plant recovery (Cheng et al, 2004; Martins et al, 2015; Tan et al, 2017). Even explant medium replacements for post-transformation culture do enhance the efficiency of transformant rice recovery (Sood et al, 2011) which depend on the genotype. Therefore, success in transformation should be followed by identifying an appropriate explant/tissue with the maximum *in vitro* regeneration capacity, optimization of gene transfer system/parameters, tailoring regeneration, selection/ screening analysis and recovery of transgenic plants as in Fig. 2. In tissue culture system for rice transformation, the prerequisite is the discovery of embryogenic cells that are accessible to the gene transfer action and can retain the regeneration ability by cell proliferation and permit selection of the transformants. Whereas a high division ratio of cells from micropropagation does not automatically indicate regenerable cells.

Gene transfer constraint

Based on experimental experience on recalcitrant rice varieties, approaches to optimize transformation protocols have been suggested. It is significant to first optimize all tissue culture stages toward increasing the accessibility of cells prior to developing a state for non-lethal gene transfer system. For *Agrobacterium* transformation and biolistic, it is significant to establish such culture and regenerable conditions for efficient gene integration, expression and final regeneration of transformant. With all that effort to optimize rice transgene integration, many species and their individual varieties are still less amenable to genetic engineering (Yookongkaew et al, 2007; Sahoo et al, 2011). As suggested by Sood et al (2011) and Liang et al (2017), the unamenable status was due to the plant genome organization towards T-DNA transfer and it is not overruled. This limitation is due to the biological disparity of the plant genetic makeup as well as lesser understanding of the interaction proceeding the transfer processes. According to Birch (1997), it is advisable to perform histological analysis of the explant leading to the success of the event. Reports indicated that the use of optimal direct transformation parameters and/or *Agrobacterium* density equally facilitate the T-DNA attachment to probable receptor proteins superficial rice explant (Hiei et al, 1994; Kumria et al, 2001).

Cell-cell recognition i.e. *Agrobacterium* attachment to plant cell surface is the primary crucial step in gene transfer. Apart from the pathogen-host receptors, the *Agrobacterium* chromosomal encoded products are required for the streamline attachment. Lack of receptor protein(s) from either site may result in failure of the attachment processes and regulation activation of the foreign gene (Lippincott and Lippincott, 1978). Low attachment frequency is obtained in cereals as a result of improper binding function of the receptors (Hiei et al, 2014; Tan et al, 2017). The parameter condition or bacterial density

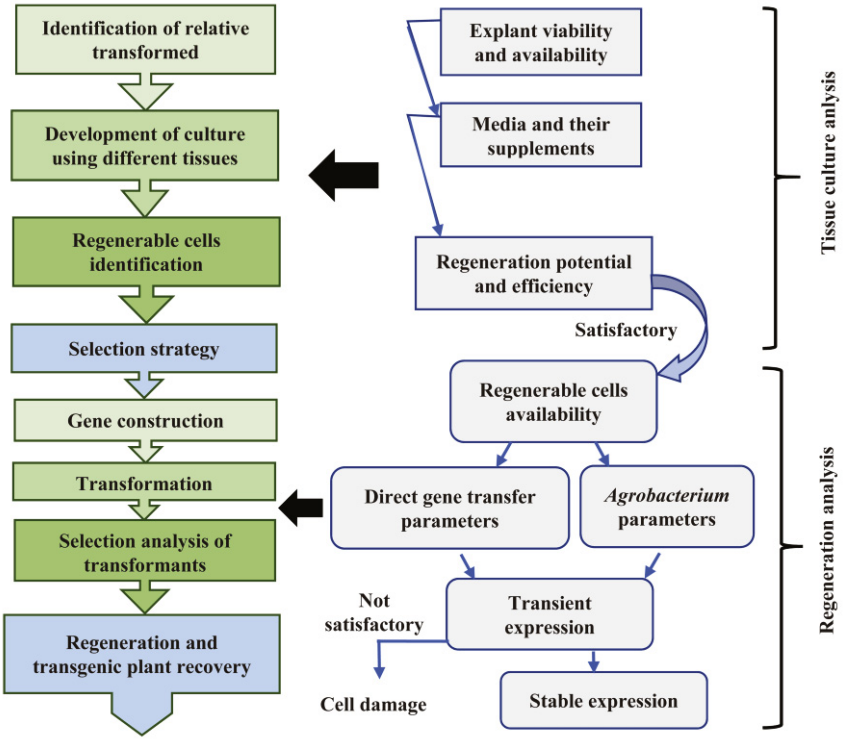

Fig. 2. Generalized strategy for tissue culture, transformation system and regeneration of transgenic rice.

varies from flora to flora and variety to variety. It is necessary to use optimum condition or concentration of *Agrobacterium* in the recalcitrant plant like *indica* rice to enhance T-DNA transfer by activating the *vir*-genes (Zaidi et al, 2006; Sahoo et al, 2011; Zuniga-Soto et al, 2015). In contrast, reports indicated the demerit of higher density towards lowering the plant recovery by damaging the plant cells and diminishing stable transformation. However, little information on the DNA forms that may upsurge the stable transformation frequency is available.

Opine producing ability of the *Agrobacterium* strains (Table 2) is paramount in transgene integration course. Naturally, the T-DNA on Ti plasmid contains oncogenes, octopine and opine catabolism that activates its expression in plant cells, suggesting that plant cell transformation by *Agrobacterium* is feasible after the development of opine-producing gall tumours (Binns and Campbell, 2001). The superiority of infectivity of nopaline producing strains over the others (octopine and the non-opine producing strains) based on the diverse role of *vir*-genes on the plasmid has been reported (Hansen et al, 1994, 1997; Park et al, 2015). Equally, chemotactic movement of *Agrobacterium* towards *vir*-gene inducers is also a factor of consideration and well debated. Both dicots and monocots are shown to exude chemoattractant, though susceptible cell recognition, chemotaxis and attachment may not be the actual limiting factors in rice *Agro*-mediated transformation. In addition, suitable choice of Ti plasmid for specific gene transfer is a valuable gadget in rice transformation analysis (Ashby et al, 1987). The vector is an important instrument with features that aid in the integration and expression of the novel gene(s) during mediated transformation. The Ti plasmid and its components plays a crucial role in gene transfer and transformant development (Hiei et al, 1994). Irrespective of transformation technique, the use of monocot-specific promoter(s), reporter and selectable marker genes by insertion of intron into the specific coding region of the plasmid are essential approaches, which enhances the efficacy of the transformation system (Cheng et al, 2004; Streatfield, 2007; Mann et al, 2012). Cauliflower mosaic virus 35S (CaMV 35S) promoter, β-glucuronidase (*GUS*) reporter gene and *hygromycin* phosphotransferase (*hpt*) marker are desired trait or characteristic of plasmid for gene transfer in rice (Hou et al, 2015). Additional promoters are equally used to advance the transfer of gene including enhanced 35S (*E35S*), rice actin (*Actl*) and ubiquitin (*Ubi*), which also enhances the transgene expression (Sood et al,

2011; Koetle et al, 2017).

Transgene integration and its expression constraint

In monocots, many blocks prevent the T-DNA integration (Bundock and Hooykaas, 1996; Cheng et al, 2004). The application of *Agrobacterium* is generally believed to produce a simpler integration prototype than direct gene transfer. Although both strategies result in a similar array of integration incidents, the copy number and frequency distributions differ (van der Graaff et al, 1996; Gelvin, 2000). Upon the *Agrobacterium* attachment to plant cell, signal transduction and *vir*-genes as well as transcriptional activation pathways regulate the infection activity and remain the factors influencing the gene insertion. In addition, certain secondary metabolites and growth regulators during *Agrobacterium*-infection period activate or inhibit the transgene integration.

The apparent T-DNA integration targeting a specific region has continued to be an important tool for subsequent transgene expression, but it is still indecipherable to date. As a result, many transformed plants produce mutant phenotype due to disruption of transcription units because of T-DNA insertion. Therefore, integration of transgene is necessary to be directed to a transcribed or even specific region without interruption of established genes (Sood et al, 2011; Koetle et al, 2015; Svitashev et al, 2015). *vir*-gene activation for transgene integration in rice is extremely challenging. Enhancing the process simply by optimization using phenolic compounds like acetosyringone (AS) or *vir* inducing chemical in rice transformation has been reported (Sarangi et al, 2011; Manimaran et al, 2013; Tan et al, 2017). In contrast, lower efficiency of rice transformations has been obtained in the absence of such chemical compounds. Thus, the mechanism guiding the chemical's role toward gene integration is yet to be fully understood. This is also another restraint factor causing the inefficiency of rice genetic transformation.

Initially, the inability of plant transcriptional machinery to recognize and express foreign genes is resolved by exploiting some control sequences isolated from virus (cauli flower mosaic) (Fang et al, 1989) and bacteria (*Agrobacterium*) (Koncz and Schell, 1986), which are known to be transcribed in plant cells. Continuous characterization and analyses involving either transient or stable expression of foreign genes construct in the plant have offered an insight into useful features for regulated transgene

Table 2. Transgenic rice generated via *Agrobacterium*-mediated transformation system (2010–2017), along with the bacterium strain, the vector's promoter and selectable marker used.

Variety	Sub-species	*Agrobacterium* strain	Reporter gene	Selectable marker	Transformation efficiency (%)	Reference
Kasalath	*indica*	EHA105	*CaMV35S:gus* *CaMV35S:sgfp* *CaMV35S:luc*	*CaMV35S:hpt*	66.9	Saika and Toki, 2010
Pusa Basmati	*indica*	EHA105	*CaMV35S:gus*	*CaMV35S:nptII*	26	Tripathi et al, 2010
MR219	*indica*	LBA4404	*CaMV35S:gus*	*CaMV35S:hpt* *CaMV35S:nptII*	± 35	Rahman et al, 2011
Handao 297	*japonica*	ALG1	*CaMV35S:gus*	*CaMV35S:hpt*	20	Zhao et al, 2011
Pusa Basmati, Taraori Basmati	*indica*	LBA4404	*CaMV35S:gus*	*CaMV35S:hpt*	80	Sarangi et al, 2011
IR64, CSR10, PB1, Swarna	*indica*	EHA105, LBA4404	*CaMV35S:gus*	*CaMV35S:hpt*	45	Sahoo et al, 2011
BR29, IR68899B	*indica*	EHA105	*rd29*	*rd29:hpt*	12	Datta et al, 2012
Zhenshan 97	*indica*	EHA105	*CaMV35S:gus*	*CaMV35S:hpt*	85.2	Tie et al, 2012
Nipponbare	*japonica*	EHA105	*CaMV35S:rUbi*	*CaMV35S:hpt*	1.8	Ozawa et al, 2012
IR64	*indica*	LBA4404	*CaMV35S:SUV*	*CaMV35S:hpt*	12	Sahoo and Tuteja, 2012
BPT5204	*indica*	LBA4404	*CaMV35S:gus*	*CaMV35S:hpt* *CaMV35S:nptII*	16.7	Manimaran et al, 2013
IR36	*indica*	EHA105	*CaMV35S:gus*	*CaMV35S:hpt*	99.5	Krishnan et al, 2013
Zhonghua 11	*japonica*	EHA105	*CaMV35S:gus*	*CaMV35S:hpt*	66.7	Yang et al, 2013
Samba Mahsuri (BPT-5204)	*indica*	LBA4404	*CaMV35S:rd29A*	*CaMV35S:hpt*	± 30	Ravikumar et al, 2014
MR219	*indica*	EHA101, EHA105, LBA4404	*mgfp:gus*	*mGFP:hptII*	5.8	Fook et al, 2015
JK1044R, JKRH401	*indica*	EHA105	*CaMV35S:gus*	*CaMV35S:hpt*	30	Chakraborty et al, 2016
Nipponbare	*japonica*	EHA105	*Ubi* promoter	*Ubi:hpt*	41.2	Xu et al, 2017
Zhonghua 11	*japonica*	EHA105	*CaMV35S:gus*	*CaMV35S:hpt*	± 67.4	Zhu et al, 2017
SR1, Jhelum, K332	*indica*	EHA105	*CaMV35S:gus*	*CaMV35S:hpt* *CaMV35S:nptII*	9.3	Yaqoob et al, 2017
MR219	*indica*	LBA4404	*CaMV35S*	*CaMV35S:hptII*	26.4	Tan et al, 2017
Sambha Mahsuri, Cotton Sannula	*indica*	LBA4404	*CaMV35S:gus*	*CaMV35S:nptII*	± 50	Reddy et al, 2019

expression in plants. The typical features considered in preparation of any constructs include transcriptional promoter and enhancer, transcriptional terminator and 3'-enhancer, polyadenylation signals, introns, codon usage, untranslated 5'-leader and 3'-trailer sequences (Birch, 1997). The pattern and level of expression generally differ to some extent even between rice sub-species and among varieties. Whereas, the variation with respect to the expression of the transgene may generate newly transformed lines which by chance have useful expression network or vise-versa. Such lucky or unlucky phenomenon as well as unpredictable phenotype loss in some transformed rice varieties have been recorded due to silencing of the transgene, which is always detected immediately after the transformation or from transformants field test (Matzke and Matzke, 1995). The transgene gene silencing or inactivation affects rice genetic transformation attempt leading to waste of time and resources. For that, much is needed to learn about the causes of such silencing or transient expression model. Thus, a multicopy number of transgenes have been recognized as a probable cause, signifying the demerit of direct gene transfer technique in monocot transformation (Sood et al, 2011).

Furthermore, some of promoter-promoter constructs are silenced, whereas some are invariably stably expressed (Birch et al, 1995). Therefore, it will be significant to identify the sequences within the constructs that inhibit or trigger the silencing and further develop a system to avoid the silence in transgenic rice plants. The *E35S* promoter shows maximum expression mechanism in some monocotyledons compared to ordinary *35S* (Sood et al, 2011; Koetle et al, 2017). In rice transformation, the *GUS*-foreign gene expression is improved by the *E35S* promoter. Other promoters such as *Actin*, *α-amylase* and *ubiquitin* are also reported to be a factor for reporter gene and transgene expression in transgenic cereals (Schoonbeek et al, 2015). *Agrobacterium* protein-complex interaction to monocot cytoplasm and/or nucleus is still incompletely understood. Similarly, the correlation between transient and/or stable expression of the transgene has not been thoroughly researched or comprehended. To produce positive outcome after any transformation approach by recovering the transgenic rice variety is highly encouraging, but it can only be actualized when the parameters affecting the *vir*-gene activation, T-DNA integration and expression are well understood.

Transformants screening and selection constraint

In transformation systems that generate a substantial number of nonchimeric transformants, genes conferring a phenotype allowing physical or visual screening or resistance-conferring genes are almost all utilized to recover rice positive transformants (Hiei et al, 1994; Saharan et al, 2004; Lowe et al, 2016). However, chimeric transformants generally necessitate screening rather than lethal selection to retrieve the primary transformants (Christou and McCabe, 1992). Screening together with selection is expensive and remains a necessary requirement for determining the transformation success and efficiency. Similarly, the recovery fold differs among selection procedures. For example, visual screening normally gives lower fold than the antibiotic selection because antibiotic chemicals provide a continued benefit to transformed cells (Birch, 1997). Due to the chemical resistance ability of the transgenic cells, they might have a higher proportion of multiplication and regeneration as well as facilitating the transformants recognition.

Selection of transformed rice tissue should be observed immediately after the transformation process, or a few days later. Generally, *Agro*-infected rice tissue selection is observed few days after the infection. The reporter genes and selectable marker expression alongside transgene vary according to cultivars and varieties (Jiang et al, 2003). Selection after co-cultivation of rice calli for few days reveals high expression of T-DNA and also simplifies the process (Manimaran et al, 2013). In addition, numerous selectable markers have been used in rice transformation systems. The gene for neomycin phosphotransferase (*nptII* or *neo*) was earlier used in the direct transformation of rice. The *nptII* confers resistance to amino-glycoside antibiotic *kanamycin* and can be equally used as a selective agent in protoplasts regeneration, but not embryogenic calli (Hiei et al, 1997; Cheng et al, 2004).

Hygromycin phosphotransferase (*hpt*) is the most common and effective plant selectable agent used for screening of rice positive transformants at present (Table 2). This confers resistance to aminoglycoside *hygromycin* and allows for discrimination between non-transformed, negatively transformed and successfully transformed tissues (Veluthambi et al, 2003). The recent redesigning of Ti plasmids by inserting an intron into the coding region of *hpt* gene or *GUS* reporter has really enhanced the transgene expression in transgenic rice, reducing the markers' copy number

in the transformants. The *hpt*-intron marker enables appropriate control of growth of *Agrobacterium* after the infection (Veluthambi et al, 2003; Sood et al, 2011). Whereas some selective markers are isolated and constructed to provide resistance to various commercially available herbicides. Using such markers under the control of *35S*, *E35S* or ubiquitin constitutive promoter works efficiently for screening/ selection after biolistic and *Agrobacterium*-mediated transformation (Cheng et al, 2004).

Phosphomannose isomerase positive marker for screening after the *Agro*-transformation of dicots has been shown to be effective in monocots including rice. However, the major limitation of such screening procedure is lack of universal efficiency across plant species (Opabode, 2006). A broadly applicable, sensitive and simple selection regime now exists for rice transgenes, viz. *GUS* histochemical assay (Table 2), which requires less experimentation, time as well as selection agent. *GUS* is expressed in the transformant cells, and releases benzyl-adenine, then supports tissue proliferation and regeneration (Joersbo and Okkels, 1996). This reporter gene has a major expression restriction when used in *Agrobacterium* transformation despite the use of plant promoters. Interestingly, the problem is overwhelmed by inserting intron (as mentioned above) or castor bean catalase gene intron(s) at the *GUS* site in the vector, which makes its activity limited to only transformed plant tissues and not detected in *Agrobacterium* species (Veluthambi et al, 2003).

Alternative reporter gene is found for immense application in positive selection of transgenic rice namely, green fluorescent protein gene (*GFP*) which visualize only in the living cells. Luciferase (*luc*) reporter gene requires an externally added substrate for detection, while GFP fluorescence occurs with ultraviolet light and oxygen without any externally added substrate (Vain, 2007). GFP displays the following drawbacks including low solubility in cytoplasm and meddling of cytoplasm-accumulated GFP during shoot regeneration. Phospho-mannose isomerase gene isolated from *E. coli* is also used as a selection agent in some cereals (Wright et al, 2001; Veluthambi et al, 2003). Irrespective of transformation technique, use of specific promoter, apposite reporter gene and selectable marker would improve the technique efficiency as well as simplifying the positive selection procedure. Considering all the different selectable markers and reporter genes, to date, none has been chosen or unanimously agreed as the best towards appropriate positive transformants selection in rice genetic engineering. Each has advantages and disadvantages.

Solution and future needs for rice transformation research

The first constraint to be addressed by research into rice genetic engineering is the tissue culture strategy (especially *indica* sub-species) to enrich regenerable tissue/cells that may be accessible to gene transfer. Development of a system for rightful explants (regenerable) selection is essential for achieving successful transformation. Routine embryogenic callus induction or ability to efficaciously establish micropropagation strategy is the fundamental requirement for achieving an *in vitro* plant regeneration and remains prerequisite for the genetic transformation, particularly in rice. Hence, the key to recalcitrant cultivars appears to be the development of a technique that will expose the abundant regenerable cells to a suitable gene integration treatment and expression. Events encompassing the gene transfer by both *Agrobacteria* and direct transfer approach should be clearly understood. But, there are lots of relevant questions surrounding the technique of direct gene transfer. This review suggested that many plant genes might be involved in both methods, and therefore, it is necessary to identify the plant factors contributing the T-DNA transfer and integration to better understand the underlying processes accounting for the susceptibility of rice cells to *Agrobacterium* infection.

Nowadays, optimization of transformation efficiency including *Agro*-infection, T-DNA transfer integration of transgene and regeneration of transgenic plants are the major concern in rice improvement technology. Another important goal is the development of new method(s) for predictable transgene expression without a collateral genetic damage to the plant genome. In the application of all plant transformation, the limiting process for cultivar improvement or plant physiology is usually not the transformant regeneration, but the selection requisite to eliminate the transgenic plants with collateral genetic damage (Birch, 1997). Indemnity of genetic damage and full recovery require more detailed investigation on the species cell physiology.

Equally, identifying other bacterial species (non-*Agrobacterium*) would be significant and might probably increase the transformation success of rice (Broothaerts et al, 2005). Interest exists for the use of non-*Agrobacterium* for rice genetic transformation

due to freedom-to-operate subjects that remain with *Agrobacterium* across numerous jurisdictions. *Ensifer adhaerens* (OV14) bacterium for rice transformation exhibits maximum infection competences (Zuniga-Soto et al, 2015). Further exploration of the *Ensifer*-mediated transformation and its mechanism is paramount. *Ensifer*-mediated transformation is suggested to be the next route for rice improvement due to its effectiveness, reliability and non-pathogenic (Wendt et al, 2012). Furthermore, research projects are undertaken with the possibility of producing valuable crops, commercially affordable and available, thus, researchers require support from industries. Similarly, scientists must unite in their goals and integrate legal, social, economic and practical issues for the research design.

CONCLUSIONS

Despite the less amenability displayed by different rice varieties and low efficiency of regeneration after *Agrobacterium*-mediated transformation, it is still possible to transform almost all rice sub-species (Sahoo et al, 2011; Sahoo and Tuteja, 2012; Zhao et al, 2015). The identified critical factors influencing the rice improvement via *Agrobacterium*-mediated transformation includes suitable and viable explant (callus/immature embryo/shoot apex) and *in vitro* regeneration capacity. Particularly, regeneration capacity is essential in determining the success of the experiment. Gene transfer through cell-cell recognition is the primary crucial step in genetic transformation. Foreign DNA integration into the viable plant nuclear genome as well as its expression has continued to be a major constrains. Correspondingly, selection of positive transformants and the improvement of the recovery fold remain important issues in transgenic rice screening.

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