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Transgenic Rice

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ABSTRACT

Rice is a remarkable plant and has been the staple food for nearly half of the world population as well as an excellent model plant in molecular biology and genomics. Genetic transformation has been an indispensable technique both in rice improvement and in basic studies ever since it became possible about two decades ago. Electroporation or polyethylene glycol-mediated transformation of protoplasts was employed in the beginning and followed by particle bombardment. Efficient methods employing *Agrobacterium tumefaciens* were then developed in the mid 1990s. Many of the gene transfers to rice have been mediated by *A. tumefaciens* for the last 10 years. Targets in improvement of rice crops include agronomic traits such as high yield, resistance to disease and insects, and tolerance to drought, low and high temperature, high salinity, and herbicides, modification of nutritional and quality factors such as vitamins, minerals, protein, lipid and starch, and capability of producing specific proteins and other metabolites. The scope of rice transformation experiments in basic studies is also complex, covering analysis of gene functions, assays of promoters, complementation of mutations, and tests of tissue culture protocols. Rice has especially been valuable in the examination of genes from other cereals and in the development of genomic resources like large-scale libraries of transformants with T-DNA insertions. Transformation technology has been continuously optimized to support diverse applications so that wider genotypes can be transformed at a higher efficiency. In this review, key developments in the studies of transgenic rice are reviewed with an emphasis on recent achievements.

Keywords: *Agrobacterium tumefaciens*, *Oryza sativa* L., transformation

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INTRODUCTION

Rice (*Oryza sativa* L.) is one of the three most important crops in the world along with maize and wheat. While the other two are predominantly used as feed or as food after extensive processing, rice is mostly consumed directly by humans without much industrial handling. Compared to other starchy crops, rice grains do not require long and tedious cooking steps before eating. Thus, rice was, is and will be the most valuable food crop to support the rapidly growing world population. Global rice production is currently 417 million tons from 153 million hectares of field (USDA, Rice Yearbook 2006), feeding nearly half of the world population, predominantly in developing countries. The demand is expected to increase even further in the coming decades.

Rice is one of the three plants that have been used most frequently in transformation experiments in the studies of plant molecular biology, biotechnology and genomics along with tobacco and Arabidopsis. While the other two are employed primarily as model plants, rice serves well both as a

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Invited Review

Table 1 Starting materials for transformation of rice.

Material	Target cells	Advantage	Disadvantage
Immature embryo	Scutellum cells starting dedifferentiated growth	➢ High transformation efficiency ➢ Low genotype dependency ➢ Short tissue culture period	➢ Fresh embryo needed every time ➢ Plant growth facility with good air-conditioning and strong light is needed
Callus from immature embryo	Dedifferentiated cells	➢ Better in quality than callus from mature seed	➢ Direct use of immature embryo is much more advantageous
Pre-cultured mature seed	Scutellum cells starting dedifferentiated growth	➢ Short tissue culture period ➢ Long term storage of seed possible	➢ Success has been reported in limited genotypes.
Germinating mature seed	Cells in apical meristem	➢ Short pre-treatment (imbibition) ➢ Little tissue culture period ➢ Long term storage of seed possible	➢ Limited success has been reported in limited genotypes.
Callus from mature seed	Dedifferentiated cells	➢ No special facility required for preparation of materials ➢ Long term storage of seed possible	➢ Low transformation efficiency in many genotypes ➢ Long tissue culture period

model of cereals and other monocotyledons and as a target of crop improvement. Tissue culture and gene transfer methods have been well established in rice, and the cultivation of rice in artificial environments is relatively straightforward. Because of its upright posture, a tiller of rice, which could be considered as a unit plant with all essential organs, occupies a very small area. Thus, experimental cultivation of rice is very space-efficient and could even be more so than Arabidopsis.

Diverse varieties are known in both of the two subspecies of rice, indica and japonica. The subspecies are generally grown in different areas, and each subspecies includes varieties suitable for tropical, subtropical and temperate regions. Tropical japonica varieties are often mentioned as the third group, javanica. The indica variety occupies 80% of the rice cultivation areas in the world. Indica has been considered to be recalcitrant in tissue culture and transformation compared to japonica, but these drawbacks are being overcome by various efforts. Another important feature of rice is that diverse genotypes across the species may be transformed reasonably well.

Therefore, rice is quite unique and remarkable in many ways and is being extensively studied in basic and applied sciences, in which gene transfer techniques have been a key technology. In this article, key developments in the studies of transgenic rice are reviewed with an emphasis on recent achievements.

TISSUE CULTURE TECHNOLOGY FOR RICE TRANSFORMATION

Transgenic rice was first produced by direct gene transfer to protoplasts mediated by electroporation or polyethylene glycol in the late 1980s (Klöti and Potrykus 1999), followed by development of a particle bombardment method and regeneration of transformants from the scutellum cells of immature embryos (Christou *et al*. 1991). Methods employing the soil bacterium *Agrobacterium tumefaciens* were then developed in the mid 1990s (Hiei *et al*. 1994). Callus cells induced from the scutella of mature seeds of japonica rice were inoculated with *A*. *tumefaciens*, and a large number of transgenic plants were produced. At present, both *Agrobacterium*-mediated transformation and particle bombardment are in use, and techniques requiring protoplasts or the "whisker" method (Mizuno *et al*. 2004), in which micro fibers of silicon carbide mediate gene transfer, are rarely used.

Advantages of *Agrobacterium*-mediated transformation include high efficiency of transformation, integration of small numbers of copies of T-DNA (transfer DNA) into the chromosomes, transfer of relatively large segments of DNA with defined ends and little rearrangement of T-DNA upon transformation. Therefore, *Agrobacterium*-mediated transformation soon became much more popular in rice than other techniques. Our quick survey of about 300 the recent papers, in which the transformation of rice is described, revealed that more than 80% of the studies used *A*. *tumefaciens* as a tool for gene transfer. Because *Agrobacterium*-mediated transformation intrinsically depends on the complex biological interaction between bacterial and plant cells, specific vectors with components that facilitate the transfer of DNA through this interaction are needed, and manipulation of complicated biological systems is required for optimization of the experimental protocols. On the other hand, in particle bombardment, which is purely a physical process, ordinary high-copy cloning vectors for *Escherichia coli* are sufficient, and gene construction steps may be very simple.

Starting materials used in *Agrobacterium*-mediated transformation and particle bombardment are listed in **Table 1**. There is no difference between the two techniques in this regard. Immature embryos appeared to be the materials that can be most efficiently transformed. The advantage of immature embryos was evident in the transformation of indica rice, which was much less efficient than japonica and javanica, because indica is generally quite recalcitrant in tissue culture and transformation. Successful transformation of indica was initially achieved only by using immature embryos via particle bombardment (Christou *et al*. 1991) and *via A*. *tumefaciens* (Aldemita and Hodges 1996). Recently, protocols for transformation of immature embryos mediated by *A*. *tumefaciens* was highly optimized so that diverse elite genotypes of indica were efficiently transformed and, in the highest case, more than 12 independent transgenic plants on average were regenerated from a single embryo of cultivar IR72 that had been co-cultivated with *A*. *tumefaciens* (Hiei *et al*. 2006; Hiei and Komari 2006). The use of immature embryos was also very effective in japonica, especially in high-quality, recalcitrant genotypes such as Koshihikari (Hiei *et al*. 2006). The key factors in successful use of immature embryos in both japonica and indica were 1) use of healthy, fresh immature embryos from plants in well-conditioned glasshouses, 2) optimization of media compositions, and 3) treatment of embryos before infection with heat and centrifugal forces (Hiei *et al*. 2006). It was suggested that a shorter tissue culture period may reduce so-called somaclonal variation, which could be detected in field trials of transgenic rice (Kim *et al*. 2004), and the use of immature embryos is preferable in this respect.

Although the use of immature embryos might be able to resolve much of the issue of genotype dependency of transformation protocols in rice, the limitation is that it is quite expensive in practice to create well-conditioned glasshouses with appropriated air-conditioning and supplemental lights available year round. Many laboratories cannot afford such a facility. Thus, the use of callus derived from mature seeds is still popular, although the frequency of transformation is relatively low and painstaking efforts seem to be required on a genotype-by-genotype basis. Indeed, most of the recent studies reporting improved protocols for transformation mediated by *A*. *tumefaciens* (Park *et al*. 2003; Rachmawati *et al*. 2004; Hoque *et al*. 2005; Kumar *et al*. 2005; Lin and Zhang 2005) and by bombardment (Martinez-Trujillo *et al*. 2003; Visarada and Sarma 2004) focused on optimization of preparation and handling of the callus in various genotypes. This tendency poses an important question of whether it is a right approach in the scientific community to allow such re-

mote efforts, which are substantial if combined, to be continued to establish protocols for transformation of the callus for specific genotypes one by one, when such genotypes may already be transformed efficiently by using immature embryos. It may be far more economical in terms of resources required for scientific research to establish consortiums of academic, public and private organizations, for example, to construct and manage a laboratory, which has an extensive greenhouse facility to supply good immature embryos, which is dedicated to plant transformation and which can perform all of transformation experiments for the scientists involved.

Pre-cultured mature seeds may also be used (Toki *et al*. 2006). So far, this method was used for only the japonica genotype 'Nipponbare', which shows good tissue response, but the frequency of transformation was reasonably high and the total tissue culture period may be as short as that of immature embryo methods. In another approach, apical meristems were targeted by piercing imbibed seeds of cv. 'Koshihikari' with a needle that had been dipped in a suspension of *A. tumefaciens* (Supartana *et al*. 2005). Expression of the marker gene in, detection of the transgene by PCR from and rescue of the integrated T-DNA from the progeny plants were reported, but Southern hybridization data were not presented. If this result is reproducible, the method will be very useful as the requirement for *in vitro* techniques is minimal.

In Arabidopsis, floral spray or floral dip methods of transformation (Chung *et al*. 2000), in which plants that had just started to flower were sprayed with or dipped in a suspension of *A. tumefaciens*. These methods are truly free from tissue-culture. If similar technology is developed in rice, it would be very useful. Ovules are thought to be the target of *A. tumefaciens* in Arabidopsis. It is still an open question whether it is possible to develop a technique to make *A. tumefaciens* enter the locule at the right stage in rice plants.

The molecular basis behind varietal differences in responses in callus induction and the regeneration of plants from the callus is poorly understood in any species of plants. A rare example was a finding that low nitrate reductase activity caused a poor tissue culture response in 'Koshihikari' and related cultivars (Nishimura *et al*. 2005; Ozawa and Kawahigashi 2006), and this finding led to development of improved protocols of tissue culture and transformation of these cultivars.

MOLECULAR TOOLS RELATED TO RICE TRANSFORMATION

Selection markers

The hygromycin resistance gene has been most frequently employed in rice, and other selection marker genes, such as herbicide resistance genes, have also contributed to the development of efficient transformation protocols. Choice of selective pressures and selectable marker genes is a key factor in efficient transformation, and there is always the possibility that better markers can be found. More than 20 selectable marker genes have been tested in various plants to date (Komari *et al*. 2006) and the number is growing. Thus, many candidate marker genes are available for tests in rice, and several new entries in the rice arena have been reported. The new markers include a feedback-insensitive α subunit of anthranilate synthase, which confers resistance to a tryptophan analog, 5-methyltryptophan (5MT) (Yamada *et al*. 2004) and an arabitol dehydrogenase gene, which confers capability to metabolize arabitol (LaFayette *et al*. 2005). It was recently reported that over-expression of the gene for the beta subunit of tryptophan synthase (*AtTSB1*) gave resistance to the tryptophan analog and a heavy metal in Arabidopsis (Hsiao *et al*. 2007). Thus, this gene may also be a useful marker in rice. A green fluorescent gene fused to the N-terminus of blastcidin S deaminase confers both resistance to blastcidin and fluorescence, which allows rapid identification of transformed cells expressing a high level of the transgenes (Ochiai-Fukuda *et al*. 2006). In addition, a nitrate reductase gene was an efficient selection marker for rice cultivars that showed low nitrate reductase activity (Nishimura *et al*. 2005; Ozawa and Kawahigashi 2006).

The fact that cells growing fast tend to lower the pH of the surrounding medium resulted in a technique for quick identification of transformed cells (Oreifig *et al*. 2004). An aminoglycoside-3"-adenyltransferase gene was introduced into rice cells, and the cells were cultured on a medium with a low level of streptomycin and a pH indicator. Transformants were quickly revealed by monitoring the change in color of the medium.

Site-specific recombination systems

Recombinases from phages and yeasts, such as Cre, FLP and R, which recombine specific sites, *loxP*, *FRT* and *RS*, respectively, are powerful tools to remove selection marker genes and other unneeded segments from plants. Recent studies of these systems in rice have focused on how the recombinase activity is expressed in the target cells for effective excision of target segments. For example, transgenic lines that contained the *loxP* sites were crossed with lines that expressed the Cre gene (Moore and Srivastava 2006). It was reported that callus induction from the F_1 seeds obtained between a line with the *RS* sites and a line with the R gene was an effective method for activating the recombination reaction (Toriyama *et al*. 2003). A chemically regulated Cre gene was constructed and used to recombine the *loxP* sites present in rice cells (Sreekala *et al*. 2005). A chimeric Cre recombinase, which can permeate the plant cell membrane, was created by fusing a 12-amino-acid membrane translocation sequence from the epidermal growth factor-4 of a Kaposi fibroblast with the Cre protein. Recombination between the *loxP* sites was observed in the rice cells that absorbed the chimeric protein (Cao *et al*. 2006).

Co-transformation

Another method for elimination of selection marker genes takes advantage of natural genetic segregation. Rice can be co-transformed efficiently with two T-DNA segments, one with genes of interest and the other with a selection marker gene, mediated by *A. tumefaciens*, and transgenic plants free from of the marker gene may be segregated in the progeny (Komari *et al*. 1996). This technique was used in the development of transgenic lines intended for commercialization (Maruta *et al*. 2001). Recently, a new binary vector with two T-DNA segments was tested in three elite cultivars of japonica indica, and the frequency of both the co-transformants and the transgenic plants from which marker free progeny were obtained was high (Breitler *et al*. 2004). Thus, the co-transformation technique may be efficiently employed in diverse genotypes in rice.

Reduction of transfer of "vector backbone" sequences

It had been believed for a long time that only T-DNA delimited by the border sequences had been transferred from *A. tumefaciens* to plant cells. However, integration of so-called "vector backbone" sequences was found to be not uncommon and became a serious issue especially in development of commercial transformants. Failure of the termination in the process of generation of T-DNA transfer intermediates at the left border of T-DNA appeared to be a major cause of this undesired phenomenon in rice (Kuraya *et al*. 2004). A method recently examined in rice was to place one, two or three additional copies of the left border sequences in transformation vectors, suppressing the transfer of the segment outside T-DNA to plant cells in a nearly perfect fashion (Kuraya *et al*. 2004).

Gene targeting

Gene targeting, which would allow specific modification of endogenous genes, has been a routine technique in various microorganisms and animals, but only limited success had been reported in plants. A breakthrough study was made in rice. A disrupted version of the waxy gene was delivered by *A. tumefaciens* to rice, and the endogenous allele was precisely replaced by homologous recombination (Terada *et al*. 2002). Relatively long stretches of flanking sequences and the use of strong positive/negative selection were key factors. Therefore, modification of genomes by gene targeting became a realistic option in higher plants. Further improvements and application of the technology have been discussed in recent reviews (Iida and Terada 2004; Cotsaftis and Guiderdoni 2005).

Additional virulence genes

Genes involved in the transfer of T-DNA are clustered in the virulence region in Ti-plasmids (tumor-inducing plasmids) in *A. tumefaciens*. It has been reported that additional virulence genes, such as *virB* and *virG* genes from a Ti plasmid, pTiBo542, carried by vectors improved the efficiency of transformation in plants including rice (Hiei *et al*. 1994). A recent finding in this regard was that the *virG* gene from another Ti plasmid, pTiAch5, elevated the efficiency of rice transformation when it was carried by the pGreen/pSoup dual binary vector system, in which the pGreen vector is replicated in *A. tumefaciens* by replication functions present in pSoup (Vain *et al*. 2004). Thus, the most effective gene or gene combinations may vary depending on the vectors, genotypes of rice and methods of transformation.

Matrix attachment region (MAR)

The matrix attachment region (MAR) is an element in eukaryotic genomes that mediates binding of chromatin to the nuclear matrix. Expression of foreign genes was found to be influenced by MAR elements placed nearby in various plants. Recent studies revealed that MAR elements, Rb7 and TM2 from tobacco and BP from the chicken lysozyme locus, can increase the level of and reduce variability in the expression of transgenes co-integrated with the elements in rice (Oh *et al*. 2005; Verma *et al*. 2005; Xue *et al*. 2005).

TRANSGENIC PLANTS IN RICE FUNCTIONAL GENOMICS

There is no question that transformation technology is a key tool in functional genomics. Because the rice genome was completely sequenced and mapped by the International Rice Genome Sequencing Project (2005), the role of transformation has grown even bigger. An approach exploiting transformation technology dedicated to functional genomics taken by a number of leading laboratories is the construction of large-scale libraries of T-DNA insertions in transgenic rice (Ryu *et al*. 2004; Sallaud *et al*. 2004; Johnson *et al*. 2005). As described in a recent review (An *et al*. 2005), different types of libraries have been constructed for various purposes, including 1) insertional mutagenesis, in which T-DNA is used to disrupt rice genes, 2) activation tagging, in which T-DNA with enhancer elements is used to activate adjacent genes, 3) a promoter trap, in which reporter genes without promoters are to be turned on by rice promoters, 4) an enhancer trap, in which expression of reporter genes with minimal promoters are to be boosted by rice enhancer elements, and 5) a gene trap, in which reporter genes are to be expressed as fusions with rice genes. These libraries are being utilized to clone and study various genes and regulatory elements. Databases of the sequences that flank T-DNA insertions are very useful for quick identification of transgenic plants with the desired insertions (Jeong *et al*. 2006).

Cloning of rice genes based on information from genetic maps (map-based cloning) has now become a popular practice, and transformation experiments provide key data to confirm gene functions. For example, in a project of cloning of the *Rf-1* gene (Komori *et al*. 2004), which is a fertility restorer gene for a type of cytoplasmic male sterility in rice, the location of the gene was first narrowed down to a segment of 76 kb in the genome by fine genetic mapping experiments, and a series of overlapping fragments from this region were transferred to male sterile rice. The *Rf-1* gene was eventually identified from a fragment that restored the fertility of the transformants. This approach is quite useful in the isolation of genes from quantitative trait loci (Nishimura *et al*. 2005; Ozawa and Kawahigashi 2006). In this context, genomic libraries constructed by using vectors, such as BIBAC and TAC, that can accommodate very large DNA fragments and can be used to transform rice are valuable resources (He *et al*. 2003; Xu *et al*. 2004).

Another simple application of transformation technology is the examination of phenotypic effects by cloned genes in transgenic rice. The highly efficient capability of rice transformation may be exploited to screen a large number of genes for useful phenotypic effects in transgenic rice. An attempt was made to combine a high-throughput rice transformation system generating tens of thousand of transgenic plants annually and an automated plant measuring setup, and a number of lead genes for improved quantitative traits, such as an increased number of seeds, have been identified (Reuzeau *et al*. 2006).

TRANSGENIC PLANTS IN RICE MOLECULAR BIOLOGY

Because genetic transformation is one of the most important basic tools in molecular biology, countless numbers of transgenic rice plants are being produced daily in various studies. Typically, the effects of over expression or regulated expression of genes from rice or other organisms or down regulation of internal rice genes are being examined in transgenic rice. Examples of the studies in various areas of research are cited in **Table 2**. It is clear at a glance at the citations that every single important issue in plant science is continuously addressed in rice. In a sense, rice in monocotyledonous plants is the counterpart of Arabidopsis in dicotyledonous plants, but the outcomes of research on rice mean much more because they have direct links to the breeding of rice as an important crop.

Transgenic plants are also useful in the studies on promoters. The scopes of studies on promoters are often broad, covering the analysis of expression patterns and the regulation of rice genes, investigation of similarity or difference in activity and regulation of promoters across species, tests of promoters for engineering of rice and dissection of *cis* and *trans* regulatory elements for promoters (Suzuki and Burnell 2003; Wang and Oard 2003; Koyama *et al*. 2005; Li *et al*. 2005; Nomura *et al*. 2005). Rice often plays a role as a surrogate species for plants recalcitrant in transformation.

EFFORTS TO DEVELOP USEFUL TRAITS IN TRANSGENIC RICE

Extensive efforts to develop transgenic traits for crop improvement and other purposes are being concentrated on rice to date. Targeted traits include disease resistance, insect resistance, tolerance to abiotic stresses, improvement in rice grain quality, nutritional fortification and capability to produce useful substances. Recent developments are discussed trait by trait below. Although an increase in crop yield is, of course, one of the most desired traits, no sub-heading for yield increase is provided here. Crop yield is an accumulated result of multiple, complex biological processes, and, in a sense, the ultimate goal of all agronomic traits is in an increase in yield. Thus, related studies are mentioned elsewhere in this article with more specific key words, such as photosynthesis, flow of carbons, phytohormones, etc.

Table 2 Examples of genes tested in transgenic rice in basic studies.

Research area	Gene	Origin	Expression	Gene effect	Reference
Starch synthesis	Mutant ADP-glucose pyrophosphrylase insensitive to allosteric regulation	*Escherichia coli*	Endosperm specific	Increased seed weight	Sakulsingharoj *et al.* 2004
Photosynthesis	Sucrose phosphate synthase (SPS)	Maize	Constitutive	Elevated photosynthetic activity and carbon export rate under higher CO_2 level	Ono *et al.* 2003
	Phospho*enol*pyruvate carboxylase (PEPC)	Maize	Constitutive	Increased photosynthetic capacity and tolerance to photoinhibition	Jiao *et al.* 2005
	PEPC	Maize	Own promoter	Higher exudation of oxalate from roots under phosphorus-deficient conditions	Begum *et al.* 2005
	Phospho*enol*pyruvate carboxykinase (PCK)	*Urochloa panicoides*	Leaf specific	Generated a flow of carbon from CO_2 to C4 acids, and additional introduction of maize PEPC had little influence	Suzuki *et al.* 2006
Nitrogen metabolism	Ammonium transporter (*OsAMT-1*)	Rice	Constitutive	Increased uptake of ammonium and decreased biomass of shoot and root	Hoque *et al.* 2006
Phytohormone	Mutant *OsIAA3* stable in the presence of auxin	Rice	Constitutive	Auxin insensitive	Nakamura *et al.* 2006
	Gibberellin (GA) 2-oxidase	Rice	Promoter of GA biosynthesis gene	Semi-dwarf with normal flowering and grain development	Sakamoto *et al.* 2003
	Antibody against $GA_{24/19}$	Mouse	Companion cell specific	Dwarf	Tanaka *et al.* 2004
Photoperiodic flowering pathway	Homologue to *CONSTANS* in Arabidopsis	Wheat	Own promoter	Complemented deficiency in Hd1 function	Nemoto *et al.* 2003
Programmed cell death	Dihydroflavonol-4-reductase	Maize	Constitutive	Elevated level of NAD(P)H pool and prevented induced cell death	Hayashi *et al.* 2005
Morphology	Phytochrome A	Arabidopsis	Constitutive	Reduced plant height, inter-node diameter and increased grain yield	Garg *et al.* 2006
	OsRAA1	Rice	Constitutive	Reduced growth of primary root, increased number of adventitious roots and delayed gravitropic response of roots, which were similar to the phenotypes of wild-type plants treated with auxin	Ge *et al.* 2004
	TERMINAL FLOWER 1/*CENTRORADIALIS*	Rice	Constitutive	Increased number of internodes, shortened length, altered radial patterns in the elongated inter-nodes, delayed heading and caused abnormal panicle architecture	Zhang *et al.* 2005
Growth	Wound inducible MAP kinase	Pepper	Constitutive	Inhibited germination and seedling growth	Lee and Back 2005
Porphyrin metabolism	5-aminolevulinic acid synthase	*Bradyrhizobium japonicum*	Constitutive	Induced photodynamic damage	Jung *et al.* 2004
Defense responses	*N*-(hydroxycinnamoyl) transferase	Pepper	Constitutive	Production of coumaryolserotoin and feruloylserotonin	Jang *et al.* 2004
	Metallothionein	Rice	Constitutive	Increased susceptibility to pathogens	Wong *et al.* 2004
			Down regulation	Higher elicitor-induced production of hydrogen peroxide	
Interaction with bacteria	Lectin (*psl*, *gs52*)	Pea and wild-soybean (*Glycine soja*), respectively	Constitutive	Promoted rhizoial colonization of roots in rice	Sreevidya *et al.* 2005
Fertility restorer for cytoplasmic male sterility	*Rf-1a*, *Rf-1b*	Rice	Constitutive	Fertility restored by silencing mRNA for a cytotoxic peptide	Wang *et al.* 2006

Disease resistance

Disease resistance genes, which have been recently evaluated in transgenic rice, are listed in **Table 3**. The most important target appears to be rice blast caused by *Magnaporthe grisea*, and a number of genes from various origins provided elevated levels of resistance to blast. Certain genes were able to offer resistance to multiple pathogens, sometimes to both fungal and bacterial pathogens, and combinations of genes were often very effective. Unlike studies of insect resistance genes, evaluation of disease resistance is mostly conducted in greenhouses and *in vitro*.

Insect resistance

The main focus of development of insect resistant rice has been on the insecticidal proteins from *Bacillus thuringiensis* (Bt) (High *et al*. 2004). Bt genes, such as *cry1Ab*, *cry1Ac* and *cry2A*, have been exploited to create rice resistant to Yellow Stem Borer and other lepidopteran pests. The effects of these genes have been well demonstrated, and the genes were introduced into various elite cultivars (Chen *et al*. 2005; Ho *et al*. 2006). Field evaluation of the resistant lines have been widely conducted mainly in Asian countries (Bashir *et al*. 2004; Chen *et al*. 2006).

Other insect resistance genes recently evaluated in transgenic rice are listed in **Table 4**. Various approaches are being taken and the examinations have been conducted mainly in growth chambers and greenhouses so far.

Table 3 Disease resistance genes recently evaluated in transgenic rice.

Gene	Origin	Expression	Elevated resistance to	Reference
Anti fungal protein (AFP)	*Aspergillus giganteus*	Constitutive	Blast (*Magnaporthe grisea*)	Coca *et al.* 2004
		Pathogen inducible	Blast	Moreno *et al.* 2005
Cecropin A	Silk moth (*Hyalophora cecropia*)	Constitutive	Blast	Coca *et al.* 2006
Non-specific-lipid transfer protein (Ace-AMP1)	*Allium cepa*	Constitutive, or pathogen inducible	Blast, sheath blight (*Rhizoctonia solani*) and bacterial blight (*Xanthomonas oryaze*)	Patkar and Chattoo 2006
Chitinase plus ribosome-inactivating protein	Rice and maize, respectively	Constitutive	Sheath blight	Kim *et al.* 2003
Thaumatin-like protein	Rice	Constitutive	Sheath blight	Kalpana *et al.* 2006
Chitinase plus thaumatin-like protein	Rice	Constitutive	Sheath blight (the resistance level was higher than a single gene)	Kalpana *et al.* 2006
Glucose oxidase	*Aspergillus niger*	Constitutive or pathogen inducible	Blast, bacterial blight	Kachroo *et al.* 2003
Mitogen-activated protein kinase 1	Pepper	Constitutive	Blast	Lee *et al.* 2004
Selenium-binding protein	Rice	Constitutive	Blast and bacterial blight	Sawada *et al.* 2004
Xa21	Rice	Own promoter	Bacterial blight	Narayanan *et al.* 2004; Zhai *et al.* 2004
Non-host resistance gene	Maize	Own promoter	Bacterial blight	Zhao *et al.* 2005
Mycotoxin detoxifying enzyme (lactonohydrolase)	*Clonostachys rosea*	Constitutive	*Fusarium* species	Higa-Nishiyama *et al.* 2005
Nucleocapsid protein	Rice hoja blanca virus	Constitutive	Rice hoja blanca virus	Lentini *et al.* 2003

Table 4 Insect resistance genes recently evaluated in transgenic rice.

Gene	Origin	Expression	Elevated resistance to	Reference
Bt (*cry1Ab*, *cry1Ac*, *cry2A*)	*Bacillus thruringiensis*	Constitutive	Yellow stem borer (*Scripophaga incertulas*) and rice leaf folder (*Cnaphlocrocus medinalis*)	Bashir *et al.* 2004; Chen *et al.* 2005; Chen *et al.* 2006; Ho *et al.* 2006
Lectin (GNA)	Snow drop (*Galanthus nivalis*)	Constitutive	Whitebacked planthopper (*Sogatella furcifera*)	Nagadhara *et al.* 2004
Trypsin inhibitor	Barley	Constitutive	Weevil (*Sitophilus oryzae*)	Alfonso-Rubi *et al.* 2003
Lectin (GNA) plus trypsin inhibitor	Snow drop and soy bean, respectively	Constitutive	*Nilaparvata lugens* and rice leaf folder	Li *et al.* 2005
Avidin	Chicken	Endosperm specific	Flour beetle (*Tribolium confusum*) and angoumois grain moth (*Sitotroga cerealella*)	Yoza *et al.* 2005

Table 5 Examples of abiotic stress tolerance genes recently evaluated in transgenic rice.

Gene	Origin	Expression	Elevated tolerance to	Reference
DREB1/CBF	Arabidopsis	Constitutive	Drought and high salt	Oh *et al.* 2005; Ito *et al.* 2006
Choline oxidase for synthesis of glycine betaine	*Arthrobacter pascens*	Constitutive	High salt	Su *et al.* 2006
δ1-pyrroline-5-carboxylate synthetase for synthesis of proline	Mothbean	Stress inducible	Drought and high salt	Su and Wu 2004
Trehalose-6-phosphage synthase plus trehalose-6-phosphate phosphatase	*E. coli*	Constitutive	Drought high salt and low temperature	Jang *et al.* 2003
Manganese superoxide dismutase	Pea	Stress inducible	Drought	Wang *et al.* 2005
Late embryogenesis abundance protein (LEA)	Barley	Constitutive	Drought	Babu *et al.* 2004
Calcineurin	Mouse	Constitutive	High salt	Ma *et al.* 2005
Arginine decarboxylase	*Datura stramonium*	Constitutive	Drought	Capell *et al.* 2004
N+/H+ antiporter	*E. coli* or yeast (*Schizosaccharomyces pombe*)	Constitutive	Drought and high salt	Wu *et al.* 2005 Zhao *et al.* 2006
Hsp101	Arabidopsis	Constitutive	Heat	Katiyar-Agarwal *et al.* 2003
Fertility restorer (*Rf-1*) for cytoplasmic male sterility	Rice	Own promoter	Improved fertility at low temperature	Komori and Imaseki 2005
Nicotianamine aminotransferase in mugeneic acid biosynthetic pathway	Barley	Stress inducible	Fe deficiency	Takahashi 2003

Abiotic stress tolerance

The role of rice as a model plant seems to be more significant in the studies of stress tolerance than those in insect and disease resistance, because biological responses to abiotic stresses are less species-specific and tolerance to drought and salinity is more badly desired in other cereals like maize. Quite a few genes have been recently evaluated in this area (**Table 5**). One of the hot subjects among them is in the capability of transcription factors, such as DREB/CBF (dehydration-responsive element-binding/C-repeat-binding) proteins (Ito *et al*. 2006), to turn on various defense genes. DREB/CBF genes from Arabidopsis and other species conferred on rice a strong level of tolerance to drought and salinity stresses.

Other genes which were evaluated and gave rice an elevated level of tolerance to drought and/or salinity stress are related to protectants of various cellular functions for osmotic, oxidative or other stresses. Other types of stress important in rice cultivation includes high or low temperatures and high or low concentrations of minerals and other soil components, and examples of such notable studies are cited

Table 6 Examples of genes evaluated for quality traits in transgenic rice.

Gene	Origin	Expression	Trait	Reference
Phytoene synthase (psy) plus phytoen desaturase (crtI)	psy: daffodil (*Narcissus pseudonarcissus*) or maize crtI: *Erwinia uredovora*	Endosperm	Golden Rice (production of β-carotene)	Ye *et al.* 2000; Al-Babili and Beyer 2005
Linolate isomerase	*Propionibacterium acnes*	Seed	Production of *trans*-10, *cis*-12 conjugated linoleic acid	Kohno-Murase *et al.* 2006
2S albumin	Sesame	Endosperm	High methionine and cysteine	Lee *et al.* 2003
Ferritin	Soy bean	Endosperm	High iron	Qu *et al.* 2005
Feed back-insensitive α subunit of anthranilate synthase	Rice	Constitutive	High tryptophan	Morino *et al.* 2005
Waxy	Rice	Down regulation	Low amylose evaluated in field	Liu *et al.* 2005
Xylanase	*Clostridium thermocellum*	Constitutive	Better digestibility by animals	Kimura *et al.* 2003
Thermostable amylopullulanase	*Thermoanaerobacter ethanolicus*	Endosperm	More efficient processing	Chiang *et al.* 2005

Table 7 Examples of useful proteins produced in rice.

Protein	Origin	Expression	Application	Reference
Interleukin 11	Human	Constitutive	Production of therapeutic protein	Lee *et al.* 2005
Granulocyte-macrophage colony-stimulating factor	Human	Constitutive	Production of therapeutic protein	Lee *et al.* 2005
Interferon-γ	Human	Cell culture	Production of therapeutic protein	Chen *et al.* 2004
Lysozyme	Human	Endosperm	Production of therapeutic protein	Hennegan *et al.* 2005
Serum albumin	Human	Cell culture by sugar starvation inducible promoter	Production of therapeutic protein	Huang *et al.* 2005
α-1-antitrypsin	Human	Cell culture	Production of therapeutic protein	McDonald *et al.* 2005
T cell epitopes	Pollen of Japanese cedar (*Cryptomeria japonicum*)	Endosperm	Edible vaccine	Takagi *et al.* 2005
Lactoferrin	Human	Endosperm	Supplement to infant food	Fujiyama *et al.* 2004

in **Table 5**.

Herbicide resistance is now a very important agronomic trait in transgenic maize and soybean. Various herbicide resistance genes have also been tested in rice. For example, a series of mammalian P450 genes were expressed in rice and several classes of herbicides were detoxified by the proteins produced by transgenic plants (Kawahigashi *et al*. 2005).

Quality traits

One of the epoch-making events in rice biotechnology was the creation of "Golden Rice" (Ye *et al*. 2000), which contains a substantial amount of β-carotene, a precursor of vitamin A, in the endosperm. The prototype of Golden Rice plants expressed bacterial phytoene synthase (PSY) and carotene desaturase (CRT1), and other genes have been employed in the transgenic rice produced later. Golden Rice is expected to help alleviate vitamin A deficiency, from which as many as 500,000 people are suffering in the world. Significant improvement was made with respect to the amount of carotenoids, the genotypes of rice, removal of the marker gene and other aspects, and some of the lines have already been evaluated in the field and appear to be close to the farmers' field. However, as discussed in a recent review (Al-Babili and Beyer 2005), there still appears to be a long way to go with tough regulatory and commercial hurdles before the lines are actually released as new varieties.

Other approaches to fortify rice nutritionally include modification of fatty acids, enrichment of specific amino acids, and higher accumulation of iron (**Table 6**). Modification of carbohydrates and expression of enzymes to help degrade carbohydrate have also been tested for better eating quality, higher digestibility or more efficient processing.

Production of proteins for pharmaceutical and industrial applications

Plants could be an efficient bio-reactor to produce proteins. Protein production in plants may be very inexpensive, and proteins from plants are free from human/animal pathogens and other undesired contaminants. While a major disadvantage of bacterial protein production systems is that products are not glycosylated, proteins produced in plants can be glycosylated in a way similar to that in mammals. Examples of proteins produced in rice in recent reports are listed in **Table 7**. Production of a number of therapeutic proteins have been tested in rice. Some proteins were intended to be used without purification. For example, rice grains that contain foreign proteins may be eaten as an "edible vaccine" or be used as food supplement. These studies have demonstrated that certain proteins may be produced abundantly in rice without losing their biological activities.

STRUCTURE AND EXPRESSION OF THE TRANSGENES IN RICE

How foreign genes are integrated in rice chromosomes and expressed in initial transformants and their progeny is of critical importance in maximizing the value of the transformation technology in basic and applied studies. Although information available in this respect in cereals had been rather limited compared to those in dicotyledons, a number of extensive studies have recently been reported on rice.

The structure of T-DNA, especially structures of the borders of T-DNA and flanking sequences, was investigated in a large number of transgenic rice plants (Kim *et al*. 2003). It was found that 35% of the transgenic lines contained a single copy of T-DNA and that many others contained direct repeats and/or inverted repeats of T-DNA. In addition, 45% of the transformants contained the vector backbone sequences, which were caused by the failure of T-strand termination at the left border (LB) or by initiation of DNA transfer from the LB.

A strain of *A. tumefaciens* that carried two vectors with distinctive T-DNA was used to transform rice, and the integration and expression patterns of the transferred genes were traced in the progeny populations of the rice plants (Vain *et al*. 2003). It was observed that the transfer of backbone sequences was found also in 45% of the lines, multiple copies of T-DNA were often integrated at separate loci, and 15-20% of the lines contained a single T-DNA without a backbone.

Multiple copies of the same sequences in the genome are often considered a cause of gene silencing. However, it was shown that gene silencing was actually caused by aberrant transcripts derived from rearranged organizations of transgenes and not by simple duplication of sequence in "super-transformed" rice plans that had already carried si-

lenced transgenes (Yang *et al*. 2005).

Transfer of very large DNA segments is a very useful technique in rice genomics. Introduction of a 92-kb segment from wheat genome into rice was attempted, and the structures of the transgenes were analyzed by fluorescence *in situ* hybridization on extended DNA fibers (fiber FISH) (Nakano *et al*. 2005). The large T-DNA seemed to be somehow integrated in rice but was associated with extensive rearrangement by duplications, deletions and insertions.

These studies generally confirmed that patterns of T-DNA integration and expression of transgenes in rice were comparable to those in dicotyledons. Therefore, various phenomena related to the behavior of T-DNA in dicotyledons are probably relevant to cereals, and methods to cope with issues found in dicotyledons will likely be useful in cereals.

COMMERCIALIZATION OF TRANSGENIC RICE

More than 10 years have passed since commercial production of transgenic crops was started in 1996, but the release of genetically modified varieties of rice has been far behind that in front-running crops, such as soybean, maize, cotton and canola. At last, it was reported that Bt rice (*Cry1Ab*) was grown in 4000 hectares of field in Iran in 2005, which was the first commercial release of transgenic rice in the world (http://www.isaaa.org/). However, there appears to be no projection that rice will soon avidly follow the front-running crops, despite the fact that quite a few field trials of transgenic rice with traits like herbicide resistance, disease resistance, insect resistance, yield increase and production of value-added proteins are ongoing (http://www.isb.vt.edu/).

There may be several reasons for the delay of commercialization of transgenic rice. Rice is primarily a food crop and the majority of the production is directly consumed by humans. The ratio of the crop for direct consumption in rice is much higher than that in the fore-running crops, which are mainly used for feed and industrial processing. Therefore, public concerns over genetically modified crops are naturally higher for rice than for the fore-runners. This would inevitably result in a requirement for extensive testing and data collection in the evaluation of transgenic rice in food and environmental safety, increasing cost for regulatory approval and product development, which is already very high.

Major players in the development of transgenic crops are multinational agrochemical and seed companies. Although the scale of world-wide grain production of rice and maize is comparable, the rice seed market is much smaller in money value, more fragmented in terms of territories and varieties and more tightly regulated by governments than maize, making rice a much less attractive target crop for the seed industry. Therefore, public organizations have to play major roles. However, product development requires skills and experiences that are rarely found in public sector scientists. In this context, a large scale collaboration of public, academic and private organizations will be very useful, and providing funds from the public sector to private companies is a reasonable option (Al-Babili and Beyer 2005).

Technical hurdles may also be high. Lines of Golden Rice and Bt rice are probably in the most advanced stages of the field evaluation mainly in Asian countries among transgenic rice lines, followed closely by herbicide tolerant rice. Although there is no question that these lines will bring huge benefits, a single trait is not expected to resolve complicated problems. For example, the Golden Rice with the current level of β-carotene is not a simple answer to many people who are exhibiting vitamin A deficiency and are also suffering from general malnutrition. Unless plants are protected from multiple insects, a Bt trait alone cannot greatly reduce pesticide sprays. Application of herbicide tolerant rice may not be straightforward because rice cultivation practices are quite diverse. Therefore, development of a range of technologies to follow these traits is essential for wide application of new technologies in rice, but many of the attractive genes are still in the stage of basic studies.

Another issue drawing recent attention is the gene flow from transgenic rice to the environment via pollen dispersal. A typical argument is that the escape of traits, such as herbicide tolerance, to weed or wild species may cause serious problems to the environment and agriculture, e.g., wide spreading of a weed that is difficult to control. However, it is generally believed that, because of the high rate of self-pollination in rice, the possibility of gene flow from transgenic rice to non-transgenic rice in adjacent fields or weeds, such as red rice (*O. sativa*), is naturally low. In fact, a number of field trials in rice confirmed that outcrossing rates were low, less than 1.0%, even if plants were planted close to each other (Zhang *et al*. 2003; Rong *et al*. 2005). Recent reviews (Gealy *et al*. 2003; Lu and Snow 2005) discussed this issue comprehensively and concluded that the extent of gene flow from cultivated rice to weedy relatives and wild species mediated by pollen is quite low and that dispersal of many types of transgenes from rice will not be harmful to the environment. These studies addressed the necessity of conducting thorough studies of environmental effects specific to the particular transgenic plants to be released, the regions where the plants are grown and the wild species present in the regions and of developing effective programs for proper management of transgenic seed, plants and grains. Such management programs would also help prevent gene flow via seed, which is potentially quite efficient, and contamination of food chains by unwanted transgenic grains.

CONCLUDING REMARKS

Review of current scientific knowledge related to transgenic rice turned out to be an ambitious challenge. Hundreds of papers dealing with transgenic rice are being published each year in major journals, and the number is ever growing. Therefore, information from the limited number of references cited here mainly in the last 3 years and issues discussed in this review constitute a picture far from comprehensive but merely a glimpse of what is going on. Nevertheless, strong progress in rice molecular biology and biotechnology is evident.

Most of the genotypes of rice may be transformed very efficiently, and many molecular tools for efficient, precise delivery and expression of transgenes in rice are in place now. Transgenic rice is a key tool in genomics and molecular biology and has been continuously playing key roles in constructing a strong knowledge base. A significant number of useful transgenic traits have already been developed, and many others are to follow. Every important issue in plant science is addressed in studies of rice. Rice is indeed a remarkable crop characterized by the dual functions of a provider of scientific information and a staple food, and this position is unmatched by other plants with a growing mass of studies in basic and applied sciences.

Despite the great progress, we do not have an optimistic view that commercial release of transgenic rice varieties will be done on a large scale within a few years. Unfortunately, regulatory, commercial, social and technical hurdles for transgenic rice seem to be higher for transgenic plants than for some other species. However, despite the great difficulties, we do not have a pessimistic view either. The fraction of efforts, expertise and resources in the plant science community dedicated to rice is so large that all of the hurdles will eventually be overcome.

Organizing large scale collaborations or consortiums of public, academic and industrial scientists, which somewhat resemble the rice genome program, may be especially beneficial in two areas. One is for efficient, high-throughput transformation of diverse genotypes in rice, which requires well-conditioned glasshouse facilities to supply fresh immature embryos. The other is for product development and commercialization of transgenic lines, which must be driven by the public sector and requires expertise in the private sector.

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