REVIEW

Genetic manipulation of rice: from protoplasts to transgenic plants

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ABSTRACT

Protoplasts are versatile tools for genetic manipulation of plant cells. The ability of genetically modified protoplasts to differentiate into complete plants offers novel experimental systems in various fields of plant biology. Among major graminaceous species (cereals), rice is the only one in which well-established protoplast system is available. By using protoplasts as an experimental system, we have obtained and characterized various mutations some of which are being used for breeding new varieties. Somatic hybrids of rice and wild *Oryza* species were generated and cytoplasmic male sterility of an indica rice was transferred to japonica rice by cell fusion. Most importantly, an efficient method of genetic transformation was developed which opened up a new area of research in rice. Various modified genes can be now introduced and expressed in transgenic rice and various mutations were generated by introduced transposable elements, *Ac/Ds*. Initial attempts on genetic engineering of rice has also begun. These suggest that rice can be a model cereal in which genetic as well as reverse-genetic approaches can be taken for various basic and applied studies.

INTRODUCTION

It is not trivial to look back on the past even if one is not too old when the area of research involved is so rapidly progressing. Plant molecular biology is one such area. For example, when I was a graduate student at Department of Genetics in University of Wisconsin, it was still generally believed that plant DNA was not clonable because of its *unique* structure. A year after I left Madison, the first protein-coding gene of higher plants was cloned in the same building I spent for more than 5 years (Sun et al., 1981) and two years later then-a-still-mysterious genetic entity, *Activator* (*Ac*) was cloned (Fedoroff et al., 1983). Since then, we have been flooded with plant genes of known sequences and this fact tremendously influences in various forms on the way plant geneticists think about what should be and can be done.

The main subject I deal with in this article is the development of a technology

which allows one to genetically modify an economically important plant, rice, then how such technical development could change the type of study one can do and the thinking of researchers trying to approach a vast number of scientific problems by using the new experimental tools. Because we have recently published reviews (Kyozyuka et al., 1989a; Shimamoto, 1991; Kyozyuka and Shimamoto, 1992) which summarize techniques and the scope of what has been done, in this article I will attempt to present a relatively personal view of how various experiments evolved from a single important experimental method.

1. *Rice protoplast culture*

Protoplasts (Cocking, 1960) are the best plant materials used for various genetic manipulation. They are single cells, thereby, plants derived from them are not, in principle, chimeric. Because of lack of rigid cell walls, protoplasts are excellent recipients of a variety of macromolecules including nucleic acids and proteins and are able to readily fuse with each other. Because of this unique nature of protoplasts, intensive studies to efficiently isolate and culture protoplasts and to regenerate plants from protoplast-derived callus have been performed. Despite of the intensive efforts in several laboratories in the world, however, graminaceous species including rice, maize, wheat and barley which are traditionally major objectives of genetic studies were recalcitrant to various techniques of protoplast culture until recently (Vasil, 1988). In 1985, two papers were published describing successful regeneration of plants from rice protoplasts (Fujimura et al., 1985; Yamada et al., 1985). These publications prompted many researchers including our group to establish protoplast culture of rice. In our laboratory, we made efforts to reduce the time required for developing rice suspension cultures suitable for isolation of a large number of embryogenic protoplasts which are able to form plants later in the culture by combining the agarose-beads method and a nurse culture method. The use of the nurse culture, in particular, was a key to establish a routine plant regeneration for rice protoplasts. Within a year after the publication of the first successful plant regeneration from rice protoplasts, we were able to establish a highly efficient rice protoplast culture method (Kyozyuka et al., 1987; Kyozyuka et al., 1988). Our immediate application of the new methods for genetic manipulation of rice were cell fusion (somatic hybridization) and introduction of foreign genes into rice. We initiated these studies, but, we also wanted to know the nature of protoplast-derived rice plants. Because it has been well known that tissue culture-derived plants often show a variety of mutations termed somaclonal variation (Larkin and Scowcroft, 1981) and that protoplast-derived plants exhibit many variations (Shepard et al., 1980). Dr. H. Ogura then at Ishikawa college of Agriculture visited our laboratory in March of 1986 and told me that he is interested in growing our protoplast-derived rice plants in the field. Our close collaboration started then and generated important information on variations of protoplast-derived rice. Indeed, we

observed a number of variations such as short height, change of maturing date (Ogura et al., 1987). Also, we found lines with increased resistant to pathogens or those with lower amylose content in the grain. Most of the variations are heritable, suggesting that a number of mutations took place during the culture of protoplasts or plant regeneration process (Ogura et al., 1988). To understand molecular basis of these mutations, Dr. P. T. H. Brown at Max Planck Institute in Köln, Germany, examined the R1 generation plants derived from protoplasts by using 12 gene sequences as RFLP probes. The results clearly indicated that a considerable number of variations in RFLP were found in protoplast-derived rice plants whereas methylation did not appear to be a major source of variations (Brown et al., 1990). While our study to understand the spectrum and genetics of mutations found in protoplast-derived rice continued, use of some of these mutations for breeding new rice varieties has been actively pursued for the last 6 years and breeders in our group have successfully developed three new varieties which are being field tested in agricultural experimental stations in a number of prefectures.

2. Somatic hybridization (cell fusion)

In 1980s, there was a high hope that cell fusion generates a novel combination of germplasms thus resulting in new useful hybrid crops (Evans, 1983). This hope prompted a number of groups to develop techniques for cell fusion and to produce hybrids between distant species. Because successful production of somatic hybrids requires a well-established protoplast culture system, somatic hybridization was primarily applied with the *Solanacea* species until late 80's. After we developed an efficient protocol for plant regeneration from rice protoplasts, we immediately initiated the study of somatic hybridization in rice. Our first attempt was to somatically hybridize rice with barnyard grass (*Echinochloa oryzicola* Vasing) which is a weed belonging to the *Gramineae* and was known to have a high capacity of plant regeneration from cultured cells. Protoplasts isolated from these two distantly-related species readily fused by the method of electrofusion and produced a large number of hybrid calli. Morphogenesis from the hybrid calli was, however, not straightforward. Various conditions of growth regulators were examined and indolacetic acid was found to stimulate shoot formation from the calli. We managed to obtain shoots (Terada et al., 1987), however, they were not morphologically normal. The hybrid shoots looked perfectly healthy, however, they suddenly became brownish and died without exception when they reached 1–2 cm in height. Many trials with a number of medium compositions did not improve the situation. The result was interpreted to mean that there was expression of incompatibility between these two species at the level of differentiation.

Next, we used several wild *Oryza* species as parents for somatic hybridization since they carry a variety of agronomically useful characters which had not been

effectively used for rice breeding because of crossing barriers. We used four wild species, *O. officinalis*, *O. brachyantha*, *O. eichingeri*, *O. perrieri*. This time, normal-looking hybrid plants were obtained with all the four combinations, suggesting that the failure of rice-barnyard grass hybrid calli to differentiate complete plants was not due to technical reasons associated with cell fusion *per se* (Hayashi et al., 1988). All the four combinations of somatic hybrids showed intermediate morphology of the parents and grew to maturity. Seeds were set, however, only from *O. sativa*+*O. eichingeri* hybrids. It was concluded from the results of these studies that cell fusion does not necessarily overcome the cross incompatibility between species, in particular, with respect to fertility.

Our failure to produce fertile hybrids by somatic hybridization suggested that asymmetric type of somatic hybridization (Dudits et al., 1988) in which a part of genome is transferred by cell fusion should be pursued as an alternative to the symmetric somatic hybridization. Asymmetric cell fusion is divided into two types: one is asymmetric with respect to the nuclear genome, the other, the cytoplasmic genome is hybrid (cybrid). The former has been suggested to be a means to transfer multiple genes controlling agronomically useful traits into species to be improved. Indeed, we have been able to develop hybrids of *Brassica campestris* and *B. oleracea* which possess the complete genome of *B. oleracea* and a variable number of *B. campestris* chromosomes (Yamashita et al., 1989; Itoh et al., 1991). The second type of asymmetric somatic hybrids are cybrids which carry hybrid cytoplasmic genome without changes in the nuclear genome. Cybridization has been a unique tool to recombine the genomes of chloroplasts and mitochondria providing useful materials for analysis of various aspects of cytoplasmic genetics in higher plants. For rice, we tried to transfer cytoplasmic male sterile (CMS) trait carried by the mitochondria of an indica rice, Chinsurah Boro II to a male fertile japonica rice by cell fusion (Kyoizuka et al., 1989b). Protoplasts isolated from the indica rice were irradiated with X-ray prior to cell fusion to destroy nuclear genome and fused with those of a japonica rice. We were able to recover a plant showing complete male sterility whose morphological characteristics were those of the japonica parent. The subsequent analysis of the plants indicated that the majority of the mitochondrial genome was derived from the japonica parent but a part which should contain genes responsible for CMS originated from the indica parent. The CMS trait of the cybrid plant gametophytically reverts to male fertility by the action of a nuclear restorer gene, *Rf1*. Continued analysis of the cybrid plants identified a mitochondrial gene, *atp6-b*, whose expression is controlled by *Rf1*. The *atp6-b* gene which is closely associated with the CMS phenotype is posttranscriptionally regulated by processing presumably carried out by the product of *Rf-1* and by editing of the transcripts whose efficiency is dependent on the processing step (Iwabuchi et al., 1993). Thus, our analysis of the rice CMS gene illustrates the usefulness of cybrids in studies of gene expression in CMS plants.

Because many agronomically useful characters are known to exist in wild *Oryza* species, the asymmetric somatic hybridization can be a useful approach in order to transfer those into cultivated rice. The potential of asymmetric cell fusion in future rice breeding remains to be further exploited.

3. Rice transformation: development of an efficient method

Genetic transformation is one of the most versatile tools in modern biology. Functions of genes that can be readily isolated by increasing number of new molecular biological techniques can be identified by reintroduction of modified genes into organisms. Moreover, the technique offers a means to genetically alter plants and animals for numerous applied purposes.

In higher plants, genetic transformation was first achieved by the use of a natural gene transfer system possessed by *Agrobacterium tumefaciens* (Schell, 1987). Unfortunately, graminaceous species including rice, wheat, maize and barley are not the host for *Agrobacterium* infection, therefore, alternative methods had to be devised for transformation (Potrykus, 1991). As mentioned earlier, protoplasts are excellent materials to introduce foreign DNA and it was established that DNA taken up by protoplasts can be stably integrated into the host chromosome (Paszkowski et al., 1984). Therefore, successful regeneration of complete plants from protoplasts indicated, in principle, that generation of transgenic rice plants via protoplast transformation is possible. In our study to establish methods for rice transformation, the choice of marker genes for selecting transformed cells was the first problem. In most transformation studies in plants, the kanamycin resistance (Km^r) gene encoding neomycin phosphotransferase II (NPTII) was used (Schell, 1987). Thus, it was then natural to try the Km^r marker gene for rice cells. The Km^r gene driven by the 35S promoter of cauliflower mosaic virus (CaMV) was initially examined with rice protoplasts as a selectable marker gene. It was immediately found that selection with kanamycin was not very efficient in rice cells mainly due to the natural resistance of rice cells to kanamycin. Furthermore, a few transgenic plants obtained by kanamycin selection turned out to be albino. We decided to try hygromycin resistance gene (*hph*) that was then occasionally used for plant transformation. J. Paszkowski at ETH, Zurich kindly gave me a hygromycin-resistant plasmid, pGL2, to examine for rice cells. The results of initial experiments with this marker were successful, producing transformed rice cells clearly resistant to hygromycin. Plant regeneration from selected Hm^r cells was without problem, and we were able to obtain green normal transgenic rice plants (Shimamoto et al., 1989). One of the major problems in rice transformation was solved. That hygromycin resistance is a superior selection marker for rice transformation is clearly seen from Table 1 which lists fertile transgenic cereals known as of July, 1992.

Another key feature of our rice transformation protocol was cotransformation. To introduce genes which are not selectable, two methods can be applied. One is

Table 1. Fertile transgenic cereals reported in the literature

Species	Year	Method	Genes	Fertility	Reference
Rice: J	1989	PP; Elec	<i>hph</i> , <i>gusA</i>	F	Shimamoto et al.
J	1990	PP; PEG	<i>hph</i>	F	Hayashimoto et al.
J	1990	PP; Elec	<i>hph</i>	F	Tada et al.
I	1990	PP; PEG	<i>hph</i>	F	Datta et al.
J	1991	PP; Elec	<i>hph</i>	F	Davey et al.
J	1991	PP; PEG	<i>hph</i>	F	Zang et al.
I, J	1991	PB	<i>hph</i> , <i>bar</i> , <i>gusA</i>	F	Christou et al.
I	1992	PP; PEG	<i>nptII</i>	F*	Peng et al.
Maize	1990	PB	<i>bar</i> , <i>gusA</i>	F*	Gordon-Kamm et al.
	1990	PB	<i>bar hph</i>	F*	Fromm et al.
Wheat	1992	PB	<i>bar</i> , <i>gusA</i>	F*	Vasil et al.

J: japonica rice, I: indica rice, PP: protoplast transformation, Elec: electroporation, PEG: polyethylene glycol, PB: particle bombardment, *hph*: hygromycin phosphotransferase gene, *gusA*: β -glucuronidase gene, *nptII*: neomycin phosphotransferase gene, *bar*: phosphinotrium acetyltransferase gene, F: fertile, F*: fertile when crossed with wild type plants.

cotransformation in which two genes, a selectable marker (Hm^r) and another gene to be introduced, are on separate plasmids. Both plasmids are simultaneously introduced into cells and transformed cells are selected with hygromycin. Hm^r cells then selected carry another gene with a high frequency. The other method is to construct a composite plasmid carrying both a selectable marker and another gene. It turned out that frequency of cotransformation, that is the proportion of Hm^r cells which also contain another gene, is 30–50%. This makes cotransformation a convenient method to introduce a variety of genes into rice since the method does not require construction of composite plasmids each time. Another merit of cotransformation is that when two genes are integrated in separate chromosomes, the marker gene can be removed by segregation in later generations whereby transgenic plants only carrying genes of interests can be obtained. This is an important feature when gene transfer methods are applied for improvement of rice because the presence of selectable marker genes which are often derived from microorganisms is not desirable.

Over the last 4 years, a number of reports on successful generation of fertile transgenic cereals have been published (Table 1). A glance of the table immediately indicates that the establishment of routine production of fertile transgenic rice plants largely depended on the well established method for protoplast culture and subsequent plant regeneration. Among the four major cereals, rice, maize, wheat and barley, rice is the only species in which foreign genes can be routinely introduced. There are only two reports on generation of fertile transgenic maize plants (Fromm et al., 1990; Gordon-Kamm et al., 1990), one recent

report in wheat (Vasil et al., 1992) although sterile and none in barley. This indicates that rice is considered to be a model monocotyledonous plant in which genetic transformation is used as a tool to study a variety of problems in plant biology. At the same time, application of genetic engineering for rice improvement can be now attempted.

4. *Introduction and transposition of the maize transposable elements Ac/Ds in transgenic rice*

Transposable elements are powerful tools for genetic and molecular biological investigations of higher plants. Several plant transposable elements have been genetically identified and characterized at the molecular level (Döring and Starlinger, 1986). The *Ac/Ds* (*Activator/Dissociation*) family is one of the best-characterized transposable elements isolated from maize. The autonomous *Ac* element is 4564 bp long and has the 11-bp imperfect inverted repeats at the ends. The *Ac* element encodes a putative transposase. The *Ds* elements are heterogeneous group of nonautonomous elements defined by their ability to transpose by the action of *Ac* and are often deletion derivatives of *Ac*.

Transposon tagging is a powerful method to isolate genes in higher plants whose products are unknown (Gierl and Saedler, 1992; Walbot, 1992). Because rice has several features well suited for application of transposon tagging, we introduced the *Ac/Ds* elements into rice to develop a transposon tagging system in rice.

Initially, we introduced *Ac* into rice by protoplast transformation and examined its transpositional behavior in callus, transgenic plants and their progeny. Based on the results of Southern blot analysis, it was found that *Ac* actively transposes in transformed calli and transgenic plants (Izawa et al., 1991). Furthermore, sequencing analysis of the excision sites of *Ac* revealed the presence of typical footprints, suggesting that *Ac* excises in rice in a similar manner as in maize. Further analysis of behavior of *Ac* in successive generations suggests that *Ac* is germinally and somatically active for several generations after introduction into rice (Hashimoto et al., unpublished results). Next, we wanted to see if a nonautonomous *Ds* element is able to transpose by the action of *Ac* in rice. To test this, *Ds* and an immobile *Ac* transposase gene were simultaneously introduced into rice protoplasts and the behavior of *Ds* was investigated (Shimamoto et al., 1993). The analysis established that the *Ds* element is able to transpose by the action of the cotransformed *Ac* transposase gene. Typical footprints at *Ds* excision sites and duplication of 8-bp target sequence at integration sites were also detected. These results strongly suggest a two-element system consisting of *Ds* elements and the *Ac* transposase gene can be employed for the development of a gene tagging system in rice.

Based on the results of analysis of *Ac/Ds* in transgenic rice, we are now developing a tagging strategy employing combination of nonautonomous *Ds* ele-

ments and an *Ac* transposase gene fused with the CaMV 35S promoter. Rice plants carrying *Ds* elements were crossed with the plant carrying the *Ac* transposase gene. In the F_1 plants, the *Ds* elements are expected to transpose to new sites in the rice genome and some of the transposition take place in germ line cells. The resultant F_2 population will contain homozygous mutations caused by germinal transposition of the *Ds* elements in the F_1 plants. Such mutations should be stable in the absence of the *Ac* transposase gene and presumably unstable to various extents if the *Ac* transposase gene is still present. Furthermore, portion of F_2 plants which carry both *Ds* and the *Ac* transposase gene can be further grown and another cycle of tagging can be repeated. Our preliminary results on screening of seedling mutants in the F_2 progeny revealed various visible mutations such as albino, stripe, dwarf, chloria and twin (Hashimoto et al., unpublished results). The frequency of mutations detected in the F_2 progeny appears to be comparable to that found in the F_2 population originated from the cross between the normal line with a mutator (*Mu*) stock of maize (Robertson, 1980). These preliminary results suggest that *Ds* elements are effective mutagens for rice. Whether interesting genes can be isolated from these mutants remains to be seen.

5. Analysis of promoter functions using transgenic rice plants

Importance of analysis of promoter functions using various types of *in vivo* expression systems is two fold. First, understanding precise expression patterns of genes is essential for understanding regulation of gene expression. Furthermore, identification of *cis*-elements residing in the 5' regulatory regions of genes is a prerequisite for understanding interaction of various sequence motifs present in the promoter with various transcription factors which specifically interact with them. Second, it is becoming clear that controlling temporal and spatial expression of agronomically useful genes is crucial for crop improvement (Mariani et al., 1990). Among various *in vivo* expression systems used for plant gene expression, transgenic plants have advantages over other systems in several ways. Information not only on quantitative aspects of promoter function but also on temporal and spatial regulation can be obtained. For this reason, transgenic tobacco plants have been often used for analysis of promoter functions of plant genes. However, promoters derived from monocotyledonous genes, especially cereal genes, do not always exhibit correctly regulated expression in tobacco presumably due to some differences existing in gene expression machinery between monocot and dicot species (Keith and Chua, 1986; Ellis et al., 1987). This has prompted us to examine expression patterns of various promoters derived from monocot genes in transgenic rice plants. Normally, 0.8–2.5 kb fragments of promoter region are fused with the bacterial *gusA* reporter gene encoding β -glucuronidase (GUS) and fusion genes are introduced into rice and examined for their expression by histochemical GUS activity staining (Fig. 1) or by quantitative GUS assay. Such analyses have been carried out with increasing number of

Figure 1. A-E. Histochemical localization of maize *Adh1-gusA* gene expression in transgenic rice plants. All sections except for the seed were made from primary transformants. a; anther; e, embryo; es, endosperm; f, anther filament; m, mesophyll; o, ovary; p, phloem; r, rachilla; rc, root cap; s, stigma; sc, scutellum; v, vascular bundle.

- A. Mature root.
- B. Cross-section of a mature green leaf.
- C. Flower.
- D. Mature pollen grains sampled on the day of flowering.
- E. One-half of the dry mature seed.

monocot gene promoters whose activities are regulated in a variety of ways.

Table 2 lists gene promoters analyzed for expression in transgenic rice plants. The results of these studies generated interesting conclusions. First, gene

Table 2. Promoters whose functions were analyzed in transgenic rice plants

Promoter	Origin	Reference
<i>Adh1</i>	Maize	Kyozuka et al., 1991
<i>Actin1</i>	Rice	Zhang et al., 1991
<i>Cab</i>	Rice	Tada et al., 1991
Histone H3	Wheat	Terada et al., 1993
<i>rbc-S</i>	Rice	Kyozuka et al., 1993
<i>rab16A</i>	Rice	Ono et al. (unpublished results)
<i>rolC</i>	Ri plasmid	Matsuki et al., 1989
35S	CaMV	Terada and Shimamoto, 1990

promoters derived from monocot species (maize and wheat) other than rice show regulated expression in transgenic rice plants expected from previous studies or mRNA analysis in various tissues (Kyozuka et al., 1991; Terada et al., 1993). Considering the fact that rice is the only cereal in which routine transformation is possible at present, rice can be a model monocot system for studying expression of various genes in transgenic plants. Second, there are minor differences between expression patterns of promoter-*gusA* fusion genes in transgenic rice and those of corresponding genes in native plants. For instance, maize *Adh1* does not express in leaves of maize while the *Adh1* promoter-*gusA* gene is expressed in leaves of transgenic rice plants. This expression pattern is well correlated with the *Adh1* gene of rice which is actually expressed in leaves. Another example is maize *rbcS* gene, in maize, a C4 plant, the gene is only expressed in bundle sheath cells while in rice the fusion gene is only expressed in mesophyll cells. These two examples appear to suggest that when differences exist in the expression pattern of a gene in the original plant and that of the foreign gene introduced in rice, the expression pattern reflects that of the corresponding gene in rice. This raises an interesting possibility that at least part of the differences in expression pattern of genes between species comes not from sequences in the promoter region but from the presence or absence and functional divergence of transcription factors and their interactions with some specific *cis*-elements in the promoter region of the gene. This should lead to identification of species-specific transcription factors present in specific cell types of rice.

Now that transgenic rice is established as a useful tool to examine promoter activities of various monocot genes, identification of specific *cis*-elements determining regulated expression of genes is being attempted. In one instance, the *rab 16* genes (Mundy and Chua, 1988) contain two highly conserved sequence motifs termed motif I (Yamaguchi-Shinozaki et al., 1989) and motif II. Previous study demonstrated the presence of protein factors in rice extracts which specifically interact with these two motifs (Mundy et al., 1991). In collaboration with Dr. N.-H. Chua's laboratory we have been testing whether both motif I and II required for the ABA response of the gene or either one of the two motifs is

sufficient for the ABA response. Analysis of expression of a series of synthetic promoters using transgenic rice tissues suggest that both motif I and motif II are required for full response to ABA (Ono et al., unpublished results). Similar types of analysis with mutant promoter constructs are being carried out with maize *Adh1* promoter and wheat histone H3 to delineate *in vivo* functions of sequence motifs using transgenic rice plants. We hope that these will lead to better understanding of functional organization of promoter regions of genes derived from monocot species.

In the future, the type of analysis being carried out with various promoter constructs can be extended to analyses on functional interactions between defined *cis*-elements and isolated DNA binding factors which are known to specifically interact with these *cis*-elements *in vitro* (Katagiri and Chua, 1992).

6. Genetic engineering of rice for better productivity

Genetic engineering of crops for various improved traits has evolved greatly during the last several years (Fraley, 1992). However, this technology has not been applied with the most important group of crops, cereals, mainly due to lack of stable transformation procedures until recently. Rice is an exception, as described above, and genetically engineered rice plants have been recently generated in our laboratory.

Rice stripe virus (RSV) causes severe damages to japonica rice in eastern Asia including Japan. The virus is transmitted by brown planthopper and its genome is composed of 4 species of RNA each of which has single-stranded and double-stranded forms (Toriyama and Watanabe, 1989). Because, virus resistance conferred by expression of coat protein genes of a number of viruses has been demonstrated in several plant species (Beachy et al., 1991), we examined whether the same strategy can be applied with a cereal. The coat protein gene of RSV was fused with the cauliflower mosaic virus 35S promoter and introduced into rice (Hayakawa et al., 1992). In order to enhance expression of the coat protein gene, the intron derived from castor bean catalase gene was inserted in the untranslated leader region of the chimeric gene because it was previously demonstrated that the intron greatly stimulated the expression of a reporter gene in transgenic rice plants (Tanaka et al., 1990). Transgenic rice plants containing the CP gene of RSV efficiently express the coat protein. Virus resistance analysis with primary transgenic plants and their progeny plants indicated that those plants expressing the coat protein are significantly more resistant to the virus than control untransformed plants. Results on the analysis of virus multiplication in the transgenic plants indicated that the virus is not able to replicate in the coat protein-expressing plants. These results clearly established that the coat protein-mediated protection against virus infection is applicable to cereals.

In addition to the virus resistance described above, we are developing insect-resistant rice by introducing a δ -endotoxin gene of *Bacillus thuringiensis*. The

B.t. toxin gene is one of the classical examples of genes effectively utilized for genetic engineering of higher plants (Vaecck et al., 1987; Perlack et al., 1990). Rice pests, particularly yellow stemborer and leaffolder, cause serious problems in many Asian countries and various breeding efforts are being made to develop resistant varieties. Therefore, introduction of *B.t.* toxin genes into rice has a great potential for the development of insect-resistant rice. To introduce and efficiently express the *B.t.* gene in rice, we first modified the base composition of the *B.t.* gene so that its codon usage is similar to those of rice genes. Because *B.t.* toxin genes carry many codons which are not efficiently used in plants, native *B.t.* genes are difficult to express in plants. Our modified *B.t.* toxin gene has altered codons for about 67% of the nucleotides without changing amino acid composition. Transgenic rice plants carrying the modified *B.t.* toxin gene expressed the *B.t.* toxin protein which can be clearly detected on immunoblots. Our results on bioassays with leaffolders using primary transgenic plants and the progeny plants indicate that the transgenic plants were significantly more resistant to the pest than control untransformed plants (Fujimoto et al., unpublished results). This is an encouraging first step to further exploit possibilities of using *B.t.* toxin genes for pest control of rice in the future.

Starch is a major component of the rice grains and composed of amylose (α 1-4 glucan) and amylopectin (branched glucan). Japonica rice normally contains 20-25% amylose in the total starch while indica rice contains higher amylose content. The amylose content of the rice grain is thought to be one of the determinants of eating quality of rice grains.

The *waxy* locus encodes UDP glucose starch glucocyl transferase (starch granule-bound starch synthetase) primarily responsible for amylose synthesis in endosperm and the gene has been cloned and sequenced (Okagaki and Wesler, 1988; Hirano and Sano, 1991). The *waxy* mutation causes the complete lack of amylose. In order to modify the amylose content of rice grains two possibilities are envisioned: decrease of amylose by expressing antisense *wx* genes to various levels and increase by introducing sense *wx* genes. For the antisense strategy, three promoters which are known to be active at various levels in developing endosperm are fused with the antisense *wx* gene and these were introduced into rice plants. In experiments with maize *Adh1* promoter and rice *wx* promoter, amylose contents of the seeds set on primary transgenic plants varied and some were much lower than control untransformed seeds. Furthermore, the decrease in the levels of the *wx* protein produced in transgenic seeds was correlated with the decrease in the levels of amylose in the seed. Interestingly, pollen collected from the transgenic plants with *Adh*/anti *wx* gene were not stained with I₂-KI, showing the waxy phenotype. This is not unexpected because maize *Adh1* promoter is also active in pollen grains.

In the second approach, we introduced the *wx* cDNA fused with the *wx* promoter into the *wx* mutant rice. Resultant transgenic seeds indeed expressed

the *wx* gene and the *wx* phenotype was restored to the wild type. Interestingly, the amylose contents of some transgenic seeds increased considerably more than the control seeds. This could be due to the *wx* promoter used for the experiments or the use of cDNA rather than the genomic DNA. These remain to be investigated in the future.

From these *wx* studies it can be concluded that the amylose contents of rice seeds can be manipulated by introduction of variously modified *wx* genes into rice. This strategy can be further extended to other genes known to be involved in starch synthesis such as genes for the branching enzyme responsible for amylopectin synthesis and for ADP glucose pyrophosphorylase which synthesizes ADPG, the direct substrate for starch biosynthesis.

The examples of genetic engineering of rice described above clearly indicate that it is now possible to improve various agronomically important characters of rice by introduction of modified genes. The number of isolated genes which are involved in important metabolic pathways is rapidly increasing. Also, new strategies to alter various functions of plants by expressing genes of eukaryotic and prokaryotic origins have been devised. Careful identification of agronomic traits to be improved and scrutinization of available genes or clonable genes should increase the chance of improving rice by genetic engineering in the future. The new technology will undoubtedly help to improve rice in combination with traditional breeding methods.

7. *Beyond transgenic rice*

In this review, I overviewed development of rice protoplast culture and two methods of genetic manipulation of rice, somatic hybridization and genetic transformation, directly developed from successful protoplast culture. The scope of research these key technologies offers is wide and possible applications keep expanding with time. Among the experimental methods described in this review, transformation is by far the most versatile tool that can be used for various areas of research. Instead of summarizing the review, I will try to examine future direction in rice research in the area of molecular genetics that will be challenged by utilizing the technique of genetic transformation.

Identification of functions of newly isolated genes

With the progress in the techniques of gene isolation such as random cDNA cloning and cloning homologous genes by PCR with heterologous primers the number of isolated genes is rapidly increasing. However, our understanding of functions of these genes is still limited and in the near future we will be overwhelmed with a vast number of gene sequences with unknown functions.

Available mutations which might be associated with newly isolated genes will be probably small since most of existing mutations of rice are morphological mutations whose biochemical bases are difficult to imagine. Then, apart from genes

encoding metabolic steps common to higher organisms, it is likely that their functions will largely remain undetermined. One approach to identify functions of isolated genes is to introduce gene-specific antisense constructs into rice and examine their phenotypic effects. This is exactly what is called "reverse genetics". Is it realistic to do such experiments with a large number of isolated genes? We should obviously limit the kinds of genes which can be experimentally dealt with even if the methods of transformation will be considerably improved in the near future. This approach will be useful with certain groups of genes whose functions are implicated in specific biological processes of plants. For instance, genes for transcription factors carrying characteristic domains, genes presumably involved in various signal transduction pathways, genes involved in defense against various environmental stresses and pathogens and genes which may have functions in morphological differentiation of various organs. Systematic approach using transgenic plants is not impossible with careful examination of structural information on the genes. Once mutants are generated by introduction of antisense genes, they can be crossed with other mutations which are existing or newly produced by transformation. This should give information on interactions of various genes. Such approaches will be likely to be more useful than those to identify molecular identities of known mutations by various cloning methods.

Generation of random mutations by transposable elements

Lack of biochemical information of majority of existing mutations in rice (Kinoshita, 1990) and lack of integration of RFLP markers (McCouch et al., 1988) and existing mutations make it unlikely to increase our understanding of gene functions at the molecular level in rice in the near future. As opposed to the reverse genetics approach mentioned above, one possible genetic approach to overcome this problem is to generate as many mutations as possible with transposable elements. Transposable elements have been traditionally used to mutagenize specific genes in maize and *Antirrhinum majus*. Rich sources of accumulated transposon-tagged mutations are central to the strength of molecular genetics of these two species. Similarly in *Arabidopsis thaliana*, a collection of mutations generated by T-DNA mutagenesis (Feldmann, 1991) and transposon tagging are known to exist. For rice, a similar collection of mutations amenable to molecular biological analysis will be needed in the future. Of course, search for new mutations by other means should be useful. As mentioned earlier, we have demonstrated that maize transposable elements *Ac/Ds* actively transpose in rice and that highly efficient mutagenesis is possible with these introduced elements in rice. For mutagenesis experiments, introduction of other known elements into rice may be also useful, and search for endogenous transposable elements of rice will become important in the future. Without mutations, it will be more and more difficult to understand complex biological processes in the plant system.

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