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Review

Plant Transformation and Transgenic Crops

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Plant transformation techniques were first developed as analytical tools of basic research on the regulational mechanism of gene expression and gene function. Presently, they are also used as applied tools to develop valuable crops. Practical methods for plant transformation to date include *Agrobacterium* mediated, electroporation, and particle gun methods, all of which have positive and negative attributes. Most of the GMO developed so far (the first generation of GMO) have such features as improved productivity or environmental and grower-friendly advantages, so that they are beneficial mainly to growers. It is, however, predicted that the second generation of GMO is likely to be placed at the center of future development of GMO. These are more related to consumers' interests, which include benefits in health promotion and palatability for developed countries and nutritiousness and peroral immunization (vaccine) functions for developing countries.

Keywords: plant transformation, GMO, *Agrobacterium*, electroporation, particle gun

1. Introduction

A quarter of a century has passed since the first international conference on voluntary restraints on recombinant DNA techniques was held in Asilomar in 1975. Techniques have progressed remarkably during this period and have not only contributed to the clarification of life science basics, but also produced excellent applied results in both medical and food production systems. In agricultural fields, the tension in the world's balance of food supply and demand is forecast to become a serious problem because of i) a substantial increase in the world's food demand arising from the population explosion and a recent shift in dietary emphasis from plant foods to animal foods in developing countries; ii) restriction of planted acreage expansion resulting from desertification and diversion of farm land; and iii) limitation of usage of irrigation, fertilizer and agricultural chemicals to raise the yield per unit area due to increasing costs as well as environmental concerns. In developed countries, however, people are taking a greater interest in healthy foods because of the increase of diseases related to overeating and the development of an aging society. Accordingly, it is desirable to create new high quality food crops with improved productivity, wide availability, and health promoting properties. However, conventional breeding techniques are relatively time and labor consuming and have many restrictions in creating new and improved varieties of food crops. These are the reasons that the use of recombinant DNA techniques, which can alter the genetic potential of crops beyond species barriers, is increasingly being counted on to settle the world's food problem in the agricultural fields.

Some transgenic crops have already been released onto the market. The planted area of these crops has increased during the past several years. Meanwhile, the debate over the commercialization of transgenic crops has heated up both scientifically and non-scientifically. Accurate recognition and proper perspective on the related issues are, however, required for meaningful discussion and final evaluation of transgenic crops. In this review, practical methods which are commonly used to produce these crops are explained. In addition, some examples for the present and future of the crops are introduced by classifying them into first generation (beneficial to growers) and second generation (beneficial to consumers).

Abbreviation: GMO, genetically modified organisms (used interchangeably with the term 'transgenic crops' in this review)

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2. Practical methods for plant transformation

There are two important steps in producing a transgenic plant. One is a process to introduce and integrate a foreign gene into a genome in plant cells or tissues. The other is to regenerate a whole plant from the transformed cells or tissues. Methods of plant regeneration from cells or tissues were well-documented long ago as extended techniques of so-called tissue culture, so this review excludes these topics from consideration and readers should refer other literature (Dixon, 1985; Kyozuka & Shimamoto, 1991). As for methods to introduce a foreign gene, there have been two techniques attempted to date, one is biological and the other is physicochemical in which the DNA molecule is directly transferred. The biological method is performed with either *Agrobacterium tumefaciens* or *A. rhizogenes*. In the wild type of *Agrobacterium*, the former induces tumors called 'crown gall' in infected plants and the latter leads to hairy root. The former system is more common these days. Physicochemical methods for a foreign gene introduction include a polyvalent cation (ex. polyethylene glycol) method (Paskowski *et al*., 1984), an electroporation method (Fromm *et al*., 1985), a liposome method (Deshayes *et al*., 1985), a particle gun method (Klein *et al*., 1987), and a micro injection method (Schnorf *et al*., 1991). Some have been used successfully, however, only the electroporation and particle gun methods are used routinely. In this chapter the principles,

features, and utilization of the three practical methods (the *Agrobacterium*-mediated method, the electroporation method, and the particle gun method) are outlined.

i) *Agrobacterium*-mediated method

Agrobacterium tumefaciens has long been known to infect the wound of a plant and to induce crown gall disease. Many studies have been made on the infection mechanism (Hiei *et al*., 1997). *Agrobacterium* carries Ti plasmid. When *Agrobacterium* infects wounded plant cells, the virulence (*vir*) region of the Ti plasmid is activated in response to phenolic compounds like acetosyringone generated by these cells. Products encoded in the *vir* region are involved in cutting out the T-DNA region of the Ti plasmid and transferring this region to the plant cell (Fig. 1). The T-DNA region of naturally occurring Ti plasmid has genes which encode the enzymes involved in the synthesis of plant hormones, auxin and cytokinin. Consequently, the hormone balance of the plant cell is disturbed which gives rise to disordered cell division leading to tumor formation. This T-DNA region also has genes encoding enzymes involved in synthesis of some amino acid derivatives known as opines which serve the bacterium as a carbon and nitrogen source. Transferring the T-DNA region to the plant cell does not require any genes within the T-DNA, but needs 25 bp reiterated sequences located at both borders of the T-DNA (left and right borders). Accordingly, it is possible to transfer a foreign gene to a plant cell when the harmful sequence within the naturally occuring T-DNA is replaced with the desired sequence of the foreign gene.

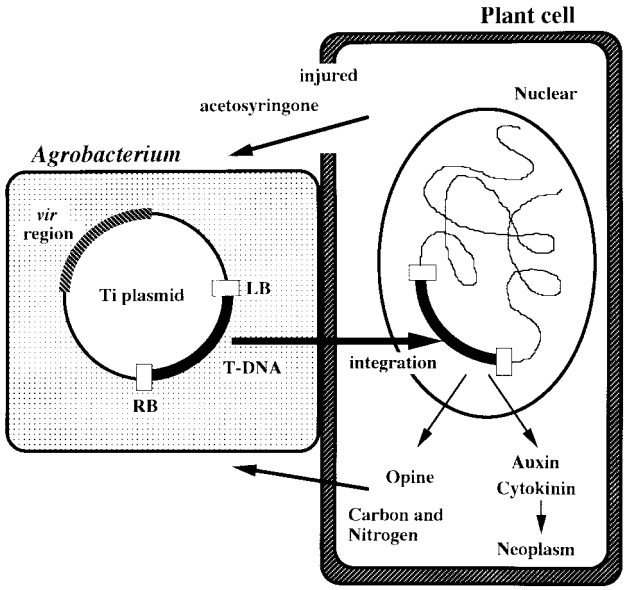

Fig. 1. Schematic representation of *Agrobacterium* infection and gene transfer. Phenolic compounds such as acetosyringone are released from wounded plant cells and activate transcription of the *vir* region of Ti plasmid. The products of *vir* genes are involved in the integration to a plant genome of the T-DNA between the 25 bp repeat sequences (right border; RB and left border; LB). Naturally occurring T-DNA genes encode enzymes involved in the synthesis of auxin, cytokinin and opines, which serve to disrupt the hormone balance and provide *Agrobacterium* with a source of carbon and nitrogen. Since the T-DNA products have no role in the T-DNA integration, foreign DNA to be inserted into the plant genome can be located between the border sequences. Arrows indicate directions of induced actions or movements of substances.

The *Agrobacterium*-mediated method has simple protocol and does not require special or expensive equipment. Usually the method induces single, or low copy number insertions of T-DNA, so that transgene rearrangement caused by recombination or homology-dependent gene silencing, which are sometimes observed in multiple copy insertions, are unlikely to happen (Table 1). In the early studies, *Agrobacterium* was thought to be infective only to some dicotyledonous plants and not to monocotyledonus plants, including most of the agronomically important crops. This aspect aided the progress of physicochemical transformation methods. In 1994, however, Japan Tobacco Inc. succeeded in getting competent monocot plant cells to *Agrobacterium* by using tissues consisting of actively dividing embryonic cells and acetosyringone inducing activation of virulence genes of Ti plasmid (Hiei *et al*., 1994). The results suggest that there may now be no host plant unable to become infected by *Agrobacterium* and that instances of plant transformation using this method will increase hereafter. However, despite the progress in this area, it is still unknown whether this method is really applicable to all plant species (Table 1).

ii) Electroporation method

With electroporation, an electrical pulse is applied to isolated plant cells with no cell wall (protoplasts) in a suspension buffer including DNA, resulting in permeabilization of the cell membrane and uptake of DNA by the cell. This method is applicable with relatively inexpensive equipment, but has many variables which may affect stable transformation efficiency and should be

Table 1. Comparison of the three plant transformation methods, *Agrobacterium*-mediated, Electroporation and Particle gun.

	Agrobacterium	Electroporation	Particle gun
Stable transformation			
Biological or Physicochemical (principle of method)	Biological (natural process of T-DNA transfer in the *Agrobacterium*-plant interaction)	Physicochemical (formation of permeable pores on plasma membrane by electric pulse)	Physicochemical (penetration of DNA with high velocity through cell wall)
Plant species applicable	Dicot and most monocots	All which can produce protoplast	All
Ease of protocol/Variables on experimental conditions	Simple/Few	Complex/Many	Simple/Some
Copy number of transgene	Single or low	Single to multiple	Single to multiple
Transgene rearrangement	Rare	Some	Some
Possibility of regeneration to chimera	Generally no	No	Yes
Cost of required equipment	None	Inexpensive	Relatively expensive
Further applications			
'in planta' transformation	Practically used	Possible	?
Transient assay	?	Useful	Very useful (tissue specific analysis is possible)
Gene targeting	?	Possible	Possible

empirically evaluated. The variables include voltage, duration of electrical pulses, spacing of electrodes, buffer composition, temperature, concentration and form of DNA, density and condition of protoplasts and so on (Table 1). Among these variables, for example, when such pulse loading conditions as field strength (voltage divided by spacing of electrodes; V/cm) and capacitance (factors affecting duration of electrical pulses; μF) are more severe (greater), transient DNA introduction efficiency to protoplasts becomes higher with the decrease of protoplast viability. Therefore, stable transformants should be obtained with compromise between these contradistinctive factors. The preparation of protoplasts is achieved by cell wall removal with mechanical or enzymatic treatment. Ease of preparation, yield, viability, and dedifferentiation/redifferentiation ability of protoplasts may be influenced by the plant's developmental stage and the type of the tissue that is used as materials. Protoplasts are assumed to be more difficult to redifferentiate than explant tissue, but are unlikely to become a chimera plant since they are made up of single cells (Table 1). The mechanism by which transferred DNA is integrated into a genome is unclear, although it is suggested that replication, ligation and recombination of the DNA may happen prior to integration (Potrykus *et al*., 1985).

iii) Particle gun method

The particle gun method depends on the delivery of microcarriers that are made of gold or tungsten particles and coated with DNA. The microcarriers are accelerated to greater than sonic speed to penetrate the plant cell wall, so that any plant species and tissues can be a target for DNA introduction (Table 1). The target tissue should, however, have potential to proliferate and result in a fertile plant in order to get a transgenic plant. Microcarriers are propelled by gun powder, helium or CO_2 gases, or an electric discharge. Compressed helium gas may be the most widely used among these acceleration sources. With this design, high pressure helium gas is rapidly released when the rupture disk bursts. The gas accelerates and conveys a macrocarrier putting the microcarriers on to a stopping plate. The macrocarrier is halted after a short distance by the stopping plate and only the microcarriers continue traveling toward the target by inertia. Size and density of microcarrier particles, as well as the velocity of microcarriers at the point of impact with the cells are suggested to be factors affecting efficient production of a stable transformant (Randolph-Anderson, 1995). Appropriate preculture or pretreatment of the target explant is also believed to be an important factor (Finer *et al*., 1999).

As mentioned above, the particle gun method is applicable to various plant tissues. This does not require the use of protoplasts which require time and labor-consuming steps for preparation and sometimes have difficulty regenerating. In these terms, it is more advantageous than the electroporation method. Nevertheless, regenerated plants via the particle gun method are sometimes a chimera which consists of both transformed and non-transformed cells. In this case, no transformant can be obtained in the next generation if the reproductive cell line of the chimera plant does not contain a foreign gene. In addition, this method requires relatively expensive equipment (Table 1).

Most of the plant transformation techniques developed so far are *in vitro* methods accompanied by a tissue culture, which requires time and effort and allows for the risk of somatic mutation. Meanwhile, an 'in planta' transformation technique, which uses an intact plant as target material for DNA introduction, is also being developed (Chowrira *et al*., 1995; Clough & Bent, 1998). This 'in planta' method is attractive for plant species in which the dedifferentiation/redifferentiation system has been established as well as for species in which this system has not yet developed, since it provides a new experimental tool. Further advances in this method are desired.

Physicochemical methods, with which various types of plasmids, DNA fragments, and labeled or modified nucleotides can be delivered, are used not only for stable transformation but also for transient assay. The particle gun method with chimeric RNA/DNA oligonucleotides was also demonstrated to be useful for gene targeting in plant cells (Zhu *et al*., 1999). That is, the physicochemical method could provide a site-directed manipulation means of chromosomal genes in plant cells as well as in bacteria, yeast, and mammalian cells and may become an ideal tool for crop improvement.

3. Overview of the first generation of GMO

The first food crop developed by recombinant DNA techniques was a shelf-life improved tomato (*Lycopersicon esculentum* M.) 'FlavrSavr' approved in the USA in 1994 (U.S. Food and Drug Administration, 1995). In this tomato, the gene encoding polygalacturonase, which is associated with the breakdown of a constituent of the tomato cell wall, was introduced inversely (antisense method). The antisense gene suppressed the sense gene expression, thus preventing the softening of ripe tomatoes. This tomato's merit is that its commercial value is retained during distribution and more flavorful tomatoes can be provided to consumers.

Subsequent to this development, herbicide tolerant and pest resistant crops were released on the market. The herbicide tolerant crops have foreign genes which provide the crops with a character that protects them from a specific herbicide. These genes are roughly divided into two types in terms of their function. For example, with the first type, the herbicide 'Glyphosate' inhibits the plant enzyme EPSPs (5-enolpyruvyl-shikimate-3-phosphate synthase) associated with biosynthesis of aromatic amino acids Phe, Trp, and Tyr (Phe and Trp are essential amino acids for human and Tyr is biosynthesized from Phe in human body: that is, human substantially lacks the biosynthetic pathway of these amino acids and the herbicide is thought to be safer). Another herbicide 'Sulfonylurea' (or Imidazolinone) inhibits the plant enzyme ALS (acetolactate synthase) associated with biosynthesis of the branched chain amino acids Ile, Leu, and Val (all essential amino acids for human). However, a bacterial or a mutated version of EPSPs and ALS is insensitive to inhibition by each herbicide, so the transgenic and the mutant crops carrying such a modified gene can fulfill the amino acid metabolic needs and survive under the herbicide (Agriculture and Agri-Food Canada, 1995a; Canadian Food Inspection Agency, 1998a). In the second type, the enzyme PAT (phosphinothricin-*N*-acetyltransferase) detoxifies 'Glufosinate' (or Bialaphos, Phosphinothricin) which controls weeds by inhibiting glutamine-synthetase and the accumulation of phytotoxic levels of ammonia in the plant. The enzyme nitrilase catalyzes the breakdown of the herbicide 'Bromoxynil' which inhibits photosynthetic reaction of a plant. Crops transformed with the gene encoding such a detoxifying enzyme can resist those herbicides (Agriculture and Agri-Food Canada,

1995b; Canadian Food Inspection Agency, 1998b). These herbicide tolerant crops do not wither under any of these herbicides, so that efficient weed control is possible in the field. The introduction of herbicide tolerant crops reportedly reduces the total amounts and number of applications of herbicides, resulting in cost reduction, yield increase, and protection of the environment.

Pest (lepidopteran, coleopteran) resistant crops have genes encoding insecticidal crystal endotoxins (CryIA(b), CryIA(c), CryIIIA) produced by a common soil bacterium, *Bacillus thuringiensis* (BT). These insecticidal crystal proteins are limited-proteolyzed in insect alkaline gut and bind to specific insect gut epithelium receptors, resulting in pore formation, loss of cations, and an osmotic pressure imbalance followed by disruption of digestive processes. Studies showed that the proteins were sensitive only to certain species of insects and were rapidly inactivated in typical mammalian acidic stomach conditions, i.e. in simulated gastric fluids. That is, the proteins are non-toxic to humans, other vertebrates, and beneficial insects (Agriculture and Agri-Food Canada, 1996a; b; c). Actually, the BT species have been used safely as environmentally acceptable foliage insecticides for more than 30 years.

Since 1994 the planted area of GMO has expanded rapidly. The reason for the fast adoption of GMO is thought to be that its economical, higher-yielding, environmental and grower-friendly advantages were well-received by growers, whether or not they were highly evaluated by consumers. The International Service for the Acquisition of Agri-biotech Applications (ISAAA) published a preview report on GMO in 1999 (James, 1999), according to which the USA stood first in the world in global planted area of GMO and occupied about three quarters (72%) of all such area in 1999, followed by Argentina and Canada, which accounted for 17% and 10%, respectively. The Organization for Economic Cooperation and Development (OECD) provides information about transgenic crops approved for planting and commercialization (OECD, 2000). They show that 61 transgenic crop products (110 lines) were approved for planting and 44 products (79 lines) approved for food or feed use in at least one country from 1992 to 1999. The majority of these crops were soybean (*Glycine max* L.), corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), and canola (*Brassica napus* L.) which represented 54%, 28%, 9%, and 9% of the total GMO planted area, respectively (James, 1999). Fifty percent of the total planted area of soybean crops and 33% of corn crops were occupied by GMO in the USA in 1999. Globally, 71% of all GMO planted areas, or 281,000 million square meters, was used for herbicide tolerant crops and 22%, or 89,000 million square meters, was used for pest resistant crops.

There are many regions on earth where the weather conditions are severe and the land is inadequate for agriculture. Research on plants growing even under such inferior conditions is in progress (Holmberg & Bulow, 1998; Kasuga *et al*., 1999). If food crops can be endowed with cold tolerance, heat tolerance, drought resistance, salt resistance, etc. and are put to practical use, they will contribute to increased and stable food production. Present GMO may be unwelcome by consumers but GMO growing in unfavorable areas may be more acceptable, particularly those living in such areas.

4. Features and examples of the second generation of GMO

In developed countries including Japan, diseases related to dietary habits, such as apoplectic stroke, heart disease and diabetes, are increasing and the society is aging. Meanwhile, the spread of infectious diseases and various essential nutrient deficiencies, including vitamin, mineral and protein deficiencies, are serious health problems in developing countries. Therefore, it is desirable that foods should be developed that prevent dietary habit-related diseases, aid against life style-related diseases or infectious diseases, and have high nutritional value. In this context, the production of the second generation of GMO has great significance; wide consumer acceptance is a prerequisite for the popularization of GMO, so those features with merit for consumers are most promising. It is forecast that the second generation of GMO is likely to be placed at the center of future development and adoption of GMO. Some examples are presented below.

i) Soy-rice

The amino acid composition of rice (*Oryza sativa* L.) is adequate for adults but not for children and infants. This is because rice protein lacks an essential amino acid, lysine. In addition, rice protein has not been found to possess any physiological function like the serum cholesterol lowering effect found in soybean protein. Moreover, rice protein does not have the functional properties for food processing that wheat and soybean proteins have. There is great potential if rice crops can be developed to incorporate lysine-rich soybean protein; this rice would have superior protein nutrition as well as hypocholesterolemic and functional properties. Rice is one of the world's staple crops and about half of the entire population of the Earth lives on this grain, so that nutritional improvement and addition of health promoting functions to rice would have great influence on improving human health conditions. Furthermore, long-stored rice and unripe green rice, of which the flavor changes or the taste is not pleasing for direct human consumption, could be used to produce more varieties of processed food, resulting in increased utility efficiency of harvested yields.

Since the major constituents of soybean and rice storage proteins have similar fundamental structures, soybean protein was assumed to highly and harmonically accumulate in rice seeds. Actually, the authors demonstrated that soybean protein could accumulate at the level of 5% of total seed proteins in endosperm, which is the major edible part of rice, using a specific promoter gene of the rice storage protein (Katsube *et al*., 1999). While the accumulated level is assumed to be almost enough for nutritional improvement of rice since the lysine content was increased by about 20% (Momma *et al*., 1999), it is not sufficient for expression of health promoting and functional properties, so that further studies on increasing the accumulation level are in progress.

ii) Iron

Studies suggest that 2000 to 3700 million people in the world are deficient in iron. Ferritin is a protein which functions to reserve iron in animals, plants, and bacteria. It has been demonstrated that oral administration of ferritin to rat is effective for anemia. The iron content of leguminous plants is high but that of cereal crops is low. Therefore, a soybean ferritin gene was introduced into rice with a promoter gene of the rice major storage protein and expressed in rice seeds (Goto *et al*., 1999). Consequently, the iron content of the edible portion of the rice was

increased threefold. This strategy is expected to have an immense contribution to overcoming the worldwide iron deficiency.

iii) β-carotene

Transgenic rice which has more β-carotene, or provitamin A, and iron has been produced by the introduction of 7 genes (Gura, 1999). Four genes among them are responsible for β-carotene synthesis and three genes for accumulation of iron in more absorbable form. Transgenic lines containing each of the β-carotene or the increased iron were produced independently; the lines were then crossed with each other to create rice containing both constituents. As little as 300 grams of the transgenic rice per day provide most of the daily vitamin A requirement. This amount is typical of the Asian diet. Vitamin A deficiency affects 400 million people and, like iron deficiency, is a serious health problem. Therefore, the transgenic rice should have a great impact. There may be anxiety, however, because some of the seven genes are derived from microbes and one has an artificial mutation.

iv) Vaccine

The spread of the infectious diseases cholera, malaria, and those from enteropathogenic microorganisms remains an important issue in developing countries. To cope with these problems, transgenic crops with a vaccine function are being developed (Arakawa *et al*., 1998; Arakawa, 1998). The first example of plant-based vaccine may be a Hepatitis B virus surface antigen expressed in tobacco (*Nicotiana tabacum* L.) plants. Also being produced in tobacco are the capsid protein of Norwalk virus causing acute enterogastritis, a surface protein antigen and a monoclonal antibody for adhesion protein of *Streptococcus mutans* which causes tooth decay. Cholera toxin B subunit (CTB) of *Vibrio cholerae*, thermolabile intestinal toxin B subunit (LTB) of enterotoxigenic *Escherichia coli* (ETEC), and Rotavirus VP6 were produced in potato (*Solanum tuberosum* L.) crops. Transgenic plants are used for foreign protein production not only for infectious diseases, but also for autoimmune diseases like insulin-dependent diabetes mellitus. Early studies depended on plants which were relatively easy to transform, such as tobacco and potato. Potato, however, must be heated before eating, so there is concern over the loss of vaccine functions. Therefore, other food crops which are eaten raw, like tomato and banana (*Musa paradisiaca* L.), will be the next target endowed with a vaccine function (Arakawa, 1998). Such crops can serve as both production and delivery systems of a vaccine.

Crops that have been approved or are in the process of approval for planting and commercialization in Japan are summarized in Table 2. GMO with such agronomical merit as herbicide tolerance, pest or pathogen resistance, and male sterility & fertility restoration are popular in Japan. In the meantime, GMO with characteristics of low allergen content, low protein content (required to produce better tasting rice wine or for patients with kidney trouble), long shelf-life, and high oleic acid content (cholesterol lowering effect) are being developed, but the number of such GMO is relatively small.

On September 28th, 1999 the Nihon Keizai Shimbun carried an article titled "Consumers keeping GMO at a distance—Western bio companies switched to value-added crops like cancer-preventive soybean etc.". Namely, they say that such companies as DuPont, Pioneer Hi-Bred International, and Novartis Seeds changed their policy for GMO development from crops with higher productivity or lower cost (the first generation) to crops with benefit for consumers (the second generation). These second generation crops include soybeans with a cancer-preventive function and some effects to counteract osteoporosis, soy-oil with a function to decrease LDL cholesterol level, and corn with increased protein content. Other examples for the second generation of GMO are crops with vitamin E accumulation (Shintani & DellaPenna, 1998) and lowered allergen content (Tada *et al*., 1996). Modified starch (Kortstee *et al*., 1998) and fatty acid (Kridl, 1998) composition and enhanced bread-making quality (Shewry *et al*., 1995) are also required to make better tasting food via modified physicochemical properties. The goal of the second generation of GMO improved in or endowed with nutritious, health promoting, and palatable characteristics is most related to consumers' interests.

Table 2. List of transgenic croups in which safety assessments have been completed or are in progress in Japan.

Crop	Introduced Feature	Safety Assessment		
		Completed	In progress	Total
Food or Feed				
Canola (14)[a)]	Herbicide tolerant	5		5
	Herbicide tolerant & MS[b)]	9		9
Corn (15)	Herbicide tolerant	4	2	6
	Pest resistant	4		4
	Herbicide & Pest resistant	1	3	4
	Herbicide tolerant & MS		1	1
Cotton (11)	Herbicide tolerant	5	1	6
	Pest resistant	2	1	3
	Herbicide & Pest resistant	1	1	2
Potato (14)	Pest resistant	13		13
	Pathogen resistant		1	1
Soybean (5)	Herbicide tolerant	1	3	4
	High oleic acid content	1		1
Sugar beet (1)	Herbicide tolerant	1		1
Tomato (11)	Long shelf life	1	2	3
	Pathogen resistant		8	8
Broccoli (2)	Herbicide tolerant & MS		2	2
Cauliflower (2)	Herbicide tolerant & MS		2	2
Cucumber (4)	Pathogen resistant		4	4
Melon (1)	Pathogen resistant		1	1
Papaya (1)	Pathogen resistant		1	1
Red bean (1)	Pest resistant		1	1
Rice (21)	Herbicide tolerant		9	9
	Pathogen resistant		4	4
	Low allergen content		1	1
	Low protein content		7	7
Strawberry (1)	Pathogen resistant		1	1
	Subtotal	48	56	104
Other Crops				
Carnation (27)	Long shelf life	6	2	8
	Flower color modified	8	11	19
Petunia (1)	Pathogen resistant	1		1
Torenia (2)	Flower color modified	2		2
Bentgrass (2)	Pathogen resistant		2	2
Chrysanthemum	Pathogen resistant		3	3
Tobacco (1)	Pathogen resistant		1	1
Zoysia Grass (2)	Pathogen resistant		2	2
	Subtotal	17	21	38
	Total	65	77	142

Summarized from data on WWW homepage on Mar./10th/2000 of Innovative Technology Division, Research Council Secretariat, Ministry of Agriculture, Forestry and Fisheries, Japan (MAFF, 2000)

[a)] The figures in parenthesis indicate the total number of transgenic lines.

[b)] MS: male sterile and/or fertility restoration.

5. Afterword

Plant transformation techniques were first developed as ana-

lytical tools of basic research on the regulational mechanism of gene expression and gene function, and now they are also used as applied tools to develop valuable crops. For the first generation of GMO, it may be allowable or even desirable that the foreign gene's expression level is low, because of the gene's features and development strategy. Most of the second generation of GMO have, however, the aim of modifying or improving features related to the quality of edible parts of crops, so that the foreign gene's high expression level is often crucial for success. Many recent studies have clarified the relationships between a *cis*-regulatory element in a promoter region and a *trans*-acting factor. In the meantime, it was often demonstrated that the exogenous gene in plants is sometimes subject to homology-dependent gene silencing (epigenetic inheritance) (Vaucheret *et al*., 1998). Therefore, the complex regulatory mechanism of gene expression and suppression in plants should be rapidly demonstrated. It is possible that the accumulation of such information might solve a problem related to expression level in transgenic crops.

As for a safety assessment of the first generation of GMO, exogenous gene products which have not been eaten by human beings and are not affirmed as 'generally recognized as safe' (GRAS), for example, antibiotic resistant gene products, are evaluated as food additives (U.S. Food and Drug Administration, 1995). The GMO and its parental traditional crop are considered to be 'substantially similar' only if the exogenous gene's function is clear and no change of characteristics between two crops is scientifically proven except for the introduced features. However, a new standard for the safety assessment of the second generation of GMO may be required. This could be necessary in crops with a significantly increased amount of a specific ingredient, with modification of a complicated metabolism system, or with protein in which the amino acid sequence is altered.

With the progress of the human genome project, nucleotide sequences of human genes are going to be determined completely. The rapid development of various analytical equipment and techniques continues to contribute to the elucidation of the function of genes. In the near future, potential risk of morbidity and mortality for each person will be predicted individually. It may become plausible to make a corresponding plan to individualize the genetic composition of food crops.

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