Towards the development and practical application of crops using new plant breeding techniques (NPBTs) such as genome editing

(Provisional Translation)

New Plant Breeding Technique Study Group

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Contents

I. Introduction

Breeding of crops has a long history of humans modifying wild or native species for many years for the purpose of realizing improved production or stable supply of food. Starting from the simple processes of humans selecting plants with useful traits (hereinafter referred to as “mutants”) found by chance in nature and reproducing them, breeding of crops has gradually evolved into much more advanced methods. Nowadays, the most mainstream of breeding techniques is breeding by crossing where desirable traits are accumulated into the cultivated crops by crossing such mutants, and the development and use of new mutation breeding techniques are progressing where mutants are artificially developed by means of radiation or chemicals. The development of breeding techniques and untiring efforts on breeding have greatly contributed to the improved agricultural productivity, stable supply of safer and higher-quality food, as well as realizing food security throughout the world.

It is widely known nowadays through the development of molecular biology in recent years that the principle of developing mutants basically relies on the genes in organisms, that the main substance of genes is DNA, and that the combinations of bases (adenine (A), thymine (T), cytosine (C) and guanine (G)) constituting DNA (hereinafter referred to as “base sequence information”) control the traits of organisms.

In recent years, new highly-sophisticated techniques have been developed to select and grow new agriculturally-useful varieties more effectively. One of them is DNA marker assisted selection, where the base sequence information of genes involved with traits useful for agricultural production is identified by decoding the genomic[1] information of crops (e.g., rice) and comparing it with the various mutants that exist in nature, and such information is used as a marker in the selection of useful varieties. Such newly developed techniques are about to be applied to breeding of various crops including rice and vegetables, and application of such molecular biological knowledge to the field of crop breeding in the future is expected to drastically increase the breeding speed and to significantly reduce the development cost. Development of breeding techniques to draw out the maximum potential of crops is essential for addressing some globally important food production issues including the necessity to increase the food production associated with the increasing global population and the fight against the global warming. Utilization of molecular biology to that end seems just a matter of course in this era.

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[1] Refers to a group of genetic information that is necessary for organisms to live. For example, the DNA of rice consists of about 0.4 billion base pairs and that of humans consists of about 3 billion base pairs.

In Europe and the USA, in addition to the DNA marker assisted selection, progress has been made on the development of "new plant breeding techniques (NPBTs[2])" that enable accurate and efficient introduction of traits useful for agricultural production, where genetic modification technologies are applied to a part of the breeding process of conventional breeding by crossing or mutation breeding.

As described in Sections II and III below, there are various techniques for NPBTs, including:

(1) those aiming at improving the efficiency of developing mutants by conventional mutation breeding (e.g., genome editing, oligonucleotide-directed mutagenesis (ODM)); and,

(2) those aiming at shortening the breeding term for conventional methods such as breeding by crossing (e.g., accelerated breeding of fruit plants, agro-infiltration).

The characteristic common to these techniques is that while genetic modification (GM) technologies are applied to a part of the breeding process, the final crop products can be free from transgenes used for genetic modification and therefore there is a possibility that plants equivalent to such engineered plants can be developed through the selection from the natural diversity or through conventional breeding by crossing, mutation breeding, etc.

For that reason, when such crops become commercialized, it will become difficult to differentiate them from crops developed by conventional breeding techniques and there arises a question whether regulations on genetically modified organisms (GMOs) shall be applied to such crops. Currently, discussions are being held overseas on the regulatory framework for NPBTs under GM regulations concerning the food and feed safety and the environmental impact (Food Standards Australia New Zealand, 2013; European Food Safety Authority, 2012a; 2012b; Lusser *et al.*, 2011).

When genetic modification technologies were used for crop breeding, the common purpose was to bestow traits that cannot be acquired by natural crossing or conventional breeding techniques, for instance introduction of an insect killing trait possessed by microbe into a crop.

Such a crop possesses a transgene originating from microbe etc. and new substances expressed by the transgene, becoming an organism that humans have never consumed or cultivated before; there is a possibility of the crop causing unexpected harmful effects on human health or wildlife. Therefore, from the viewpoint of preventing such harmful effects, national regulatory frameworks have been set to require safety assessment for each individual case and only those with confirmed safety and approval by the Government can be cultivated, imported, etc. Currently, such a system of ex-ante regulations is widely adopted in various countries in the world.

[2] New plant Breeding Techniques

The Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan is now accelerating the development of new innovative varieties that generate "strength" in Japan's agricultural and livestock products as part of its policy to realize "Aggressive Agriculture, Forestry and Fisheries", and NPBTs may become important key techniques to realize the goal, along with the DNA marker assisted selection.

Additionally, amid the situation where the transformation of agriculture, forestry and fisheries industries into growing industries is placed as an important political task for the entire Japanese Government, the "Comprehensive Strategy on Science, Technology and Innovation 2015 (Cabinet Decision on June 19, 2015)[3]" indicates the "development of next-generation breeding systems such as NPBTs" as one of the priority R&D initiatives to be promoted in the future, and the relevant ministries are jointly promoting R&D under the Strategic Innovation Promotion Program (SIP)[4].

However, on practically applying the results of such research, the important task in Japan is how to develop social understanding of crops and foods developed by NPBTs at the commercialization, while there still remain strong concerns among Japanese consumers and producers over the safety of crops and foods to which GM techniques are applied.

Additionally, while they have already started discussions on the regulatory framework for NPBTs under GM regulations in the EU etc., we also need to promote collection and analysis of scientific knowledge and experience that can form a basis of such discussions, and to hold discussions with diverse stakeholders in the future taking into account the situation of NPBT R&D overseas and the status of consideration on the regulations over NPBT in other countries. Further, there is a necessity to promote activities to internationally share such scientific findings towards achieving international harmonization on the regulatory framework for NPBTs and genetically modified organisms.

Based on such background, in October 2013, a study group consisting of experts was established within the Agriculture, Forestry and Fisheries Research Council Secretariat, aiming at gathering domestic and overseas information on NPBTs, compilation of scientific findings on the effects on the biological diversity, proper promotion of related R&D, smooth social implementation of research results, etc.

The main duty of the Study Group was to investigate what kinds of breeding techniques currently exist as NPBTs, to assess and analyze how the crops developed by taking advantage of the technological characteristics etc. of NPBTs are addressed under the current GM regulations (Food

[3] http://www8.cao.go.jp/cstp/sogosenryaku/index.html
[4] http://www8.cao.go.jp/cstp/gaiyo/sip/

Sanitation Act, Feed Safety Act and the Cartagena Act) in advance, and thereby to promote the relevant R&D in accordance with the current regulations.

However, during the considerations at the Study Group, it was indicated that (1) many NPBTs apply the latest findings of molecular biology to breeding of crops and further technical improvement and development in the future are expected, (2) in Japan, scientific assessment of GM technique-applied foods on their safety and effects on the biological diversity is in principle carried out on a case-by-case basis, taking into account the properties etc. of the engineered crops or food (product) instead of individual breeding techniques (process), and (3) assessment of food safety etc. must be done by experts in each field.

To that end, the Study Group decided only to provide scientific opinions on effects on the biological diversity that is the expertise of the Study Group members, using some R&D for which MAFF aims at practical application in the future as case studies (Chapter III).

The Study Group hopes that R&D at MAFF shall properly comply with regulations such as the Cartagena Act etc. in the future based on the points to be considered that were summarized in this Report (Chapter IV). The Study Group also hopes investigations and discussions will be enhanced by various experts including food safety experts by continuously collecting and analyzing scientific findings, obtaining information on relevant regulations overseas and by promoting international dialogue towards the harmonization of regulatory framework etc., through cooperation and collaboration with the relevant regulatory authorities including food safety aspects.

Additionally, social implementation of leading-edge science and technology such as NPBTs essentially requires promotion of interactive mutual communication with a wide variety of relevant people from the R&D stages, and it is important to reflect the voices of anticipation, anxiety and concerns from the nation on the R&D and the process of practical application. In that sense, the Study Group strongly desires that the release of this Report triggers a fostering of better understanding of the technical characteristics and regulatory issues of NPBTs by the general public and commencement of broad discussions on NPBTs and GMOs in Japan on a wide range of topics, including how NPBTs shall be utilized in the future for the purpose of revitalizing the agriculture, forestry and fisheries industry, improving the quality of life for the nation, and solving the global issues that humans may encounter in the future.

II. Trend of R&D and Regulations on NPBTs Overseas

1. Trend of R&D

In the EU, the "New Techniques Working Group (NTWG[5])" consisting of scientists etc. was established under the European Commission (EC) in 2007 in response to a consideration request by the Netherlands, and deliberations have been made on the regulatory framework for NPBTs.

As part of the deliberations, in 2011, the European Commission, Joint Research Centre (JRC), Institute for Prospective Technological Studies released a report entitled "New plant breeding techniques. State-of-the art and prospects for commercial development[6]". According to this report, currently the development and practical application have progressed for 7 techniques (techniques (1) to (7) below) centering on research institutions and private companies in the US and Europe, and:

1. The EU leads the world in the number of related scientific papers issued and the USA leads in the number of patent applications;
2. As a result of a questionnaire survey given to major biotechnology enterprises, NPBTs have been adopted by commercial breeders and the most advanced crops could reach the stage of commercialization in the short to medium term (2-3 years) if the crops produced by using these techniques are not classified as GMOs.
3. All techniques are undergoing preparations for practical application, placing emphasis on higher efficiency rather than conventional breeding and substantial reduction in the cost for developing new products. Crops resulting from most of the techniques cannot be distinguished from conventionally bred crops and detection is therefore not possible.

In 2012, the Food Standards Australia New Zealand (FSANZ) established a scientific panel[7] for the purpose of obtaining expert advice on the handling of such breeding techniques under the GM regulations. During the course of its workshops held to date, a total of 12 techniques have been introduced in addition to the 6 breeding techniques (e.g., genome editing) that were deliberated in

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[5] New Techniques Working Group

[6] Lusser *et al*. "New plant breeding techniques. State-of-the art and prospects for commercial development", http://ipts.jrc.ec.europa.eu/publications/pub.cfm?id=4100

[7] http://www.foodstandards.gov.au/consumer/gmfood/Pages/New-plant-breeding-techniques-in-the-spotlight.aspx

the EU, including the hybrid maize seed production technology (SPT[8], technique (8) below) practically applied by the US DuPont Pioneer[9].

NPBTs discussed in the EU and Australia/NZ are outlined below.

(1) Genome editing using artificial restriction enzyme

It is known that mutants like so-called "bud variations" are generated due to mutation of the gene (DNA) in plants caused by various natural effects such as UV. Nowadays, mutation breeding to artificially induce mutation by utilizing radiation or chemicals is practically applied and used in breeding and improving of crops. In such mutation breeding, mutation of DNA randomly occurs on the chromosome of plants (hereinafter referred to as "on the genome"), and the possibility of mutation occurring to the gene (DNA) involved with the target trait of breeding and improving is extremely low. For that reason, it used to take many years for humans to find useful varieties (Abe *et al.*, 2013).

Lately, "artificial restriction enzymes" that allow for accurate cleavage of specific base sequence domains of target genes (DNA) have been developed, and it has become possible to arbitrarily induce gene modification (deletion, substitution or insertion) to the targeted domain on a genome (genome editing).

By applying the genome editing to plant breeding, it would be possible to arbitrarily modify a endogenous gene that affects the flower color, plant height, etc. and thereby develop new innovative varieties in a short period of time.

There are two representative methods of genome editing using artificial restriction enzyme; one is Zinc Finger Nuclease (ZFN) and the other is Transcription Activator Like Effector Nuclease (TALEN). Both of the proteins consist of "DNA binding domain" to identify the specific base sequence information on the genome and "DNA cleavage domain (*Fok* I)" with restriction enzyme activity.

The mechanism of these techniques is that when the relevant DNA binding domain binds to the homologous section of double-stranded DNA of the organism (host) and the DNA cleavage domain has formed a dimer, the double-stranded DNA of the host is cleaved (Osakabe and Osakabe, 2013; Engstrom *et al.*, 2009) (Fig. 1).

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[8] Seed Production Technology, https://www.pioneer.com/CMRoot/pioneer/about_global/our_research/enabling_technologies/enabling_technologies_sheets/tech_spt_2014.pdf

[9] While the report of FSANZ introduced it as a technique of "Pioneer Hi-Bred International", this report follows the material by MAFF, http://www.s.affrc.go.jp/docs/commitee/diversity/130326/pdf/sankou3.pdf

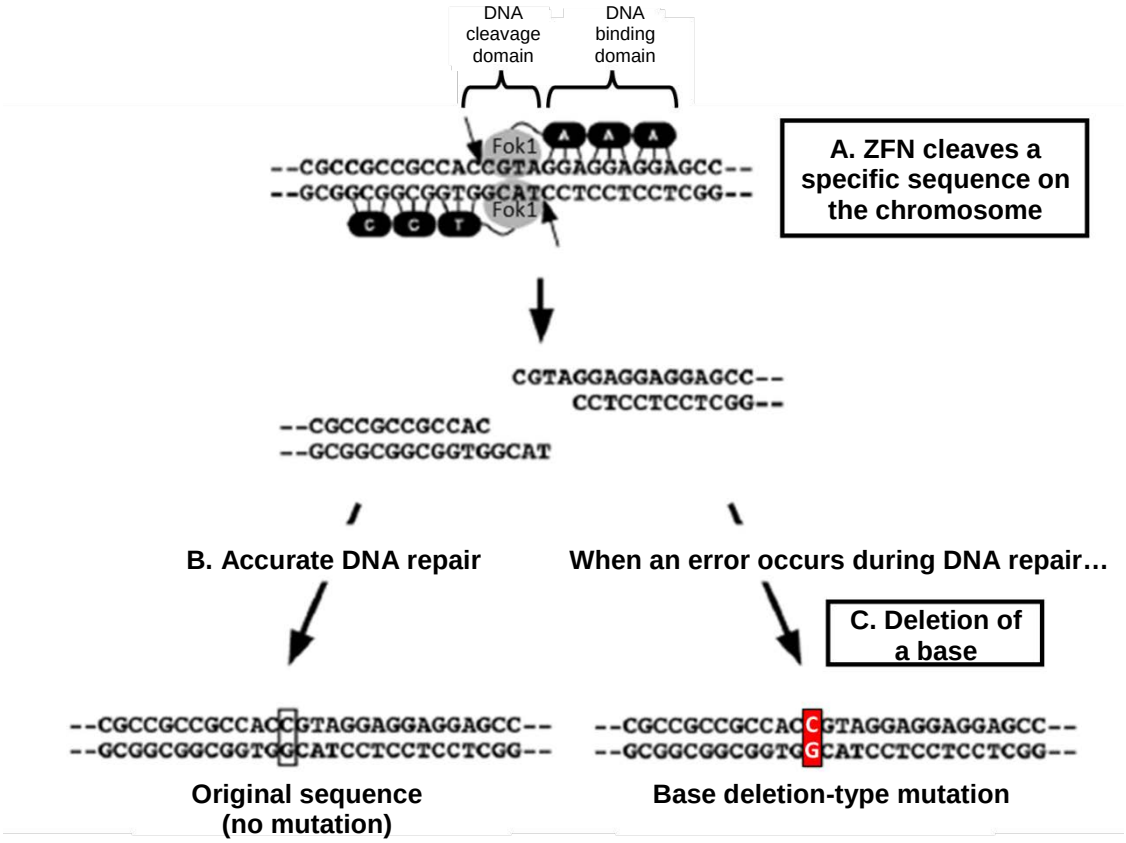

Fig. 1. Schematic of DNA cleavage by an artificial restriction enzyme (ZFN).

Additionally, recently, a technique called CRISPR[10]/Cas system has been developed, where the DNA binding domain utilizes not a protein but RNA (Sakuma, 2013).

The chance of the same base sequence existing on genome can be calculated as $1/(4^{18})$ (maximum of 1 in 70 billion), for instance, if the number of bases in the identified sequence of DNA binding domain is 18 (Fig. 1). Considering that plant genomes consist of billions of base pairs, as long as special attention is paid to the specificity of the base sequence of DNA binding domain at the time of designing the artificial restriction enzyme, it can be said that the possibility of cleaving non-targeted base sequence on the genome (so-called "off-target") is extremely low (Hsu *et al.*, 2013).

Usually double-strand DNA breaks are swiftly repaired in the cell of the host, and there are three types of genome editing that takes advantage of DNA repair (Ezura and Osawa, 2013; Porteus, 2009) (Fig. 2):

[10] Clustered Regularly Interspaced Short Palindromic Repeats

1. Expects an error occurring during the repair and random mutation (base substitution, insertion or deletion) occurring for one or a few bases (Site-Directed Nuclease 1 (SDN-1));
2. Systematically induces mutation for 1 or a few bases by artificially synthesizing a short DNA fragment (template) that is homologous to the target base sequence and introducing it along with an artificial restriction enzyme at the time of cleaving (SDN-2); and,
3. Forms a special DNA fragment at a specific domain on the genome by introducing a long DNA fragment containing a gene of several thousand base pairs not originating from a compatible same or related varieties (transgene) in a form sandwiched by sequences homologous to the target sequence (SDN-3).

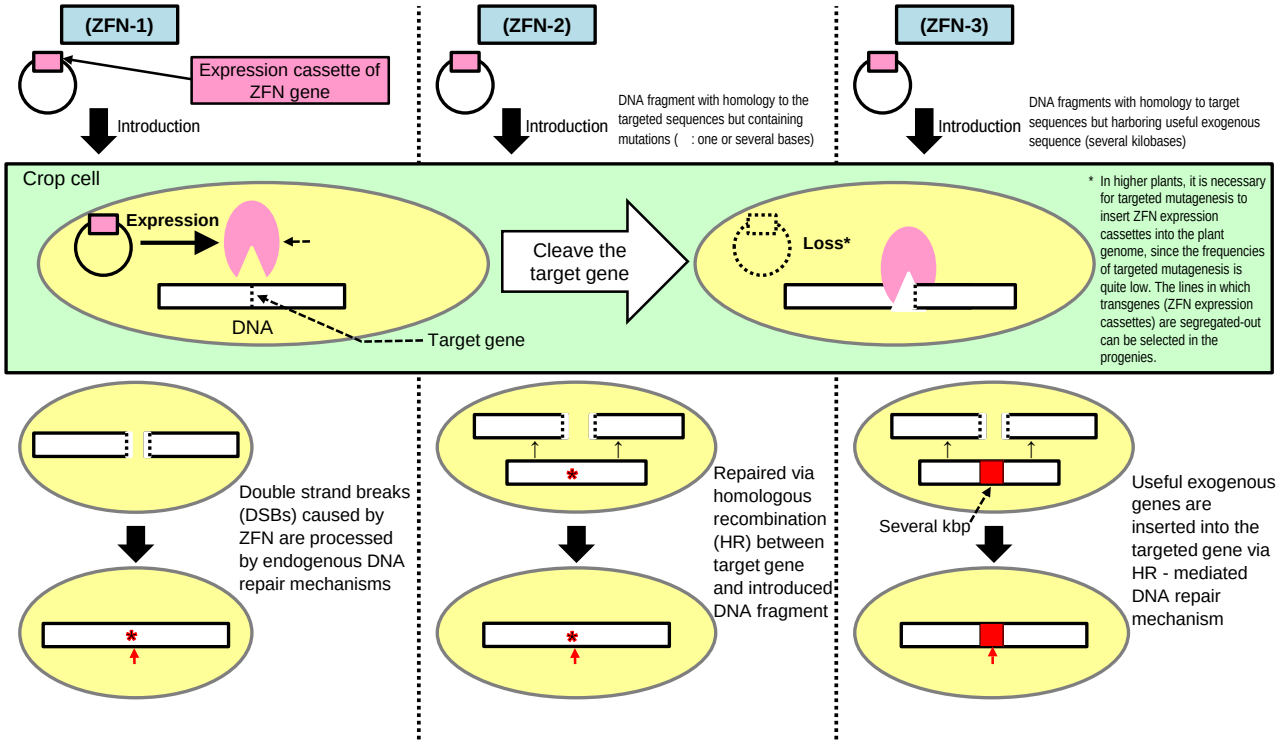

Fig. 2. Types of genome editing.

While ZFN and TALEN enable induction of such mutation to the gene (DNA) of animals by directly introducing the ZFN/TALEN protein (artificial restriction enzyme) into the cell, different methods are used for plant cells. For plants, usually, (1) the protein is transiently expressed by incorporating the gene into a vector often used for GM techniques, or (2) the gene (gene that expresses artificial restriction enzyme) is incorporated into the crop genome, the artificial restriction enzyme is expressed, mutation is induced, and then the relevant gene is removed by backcrossing with conventional varieties or other method.

(2) Oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis (ODM[11]) is a technique to induce mutation to endogenous gene of organism (crop) artificially, similar to genome editing above, and has a history of R&D over nearly 30 years.

The process of ODM is to synthesize an oligonucleotide[12] or a short single-stranded DNA fragment (length of 20-30 bases) that is homologous to the target base sequence but has mutation of about 1 base and to directly introduce it into a cell by particle bombardment etc. (Ezura and Osawa, 2013) (Fig. 3).

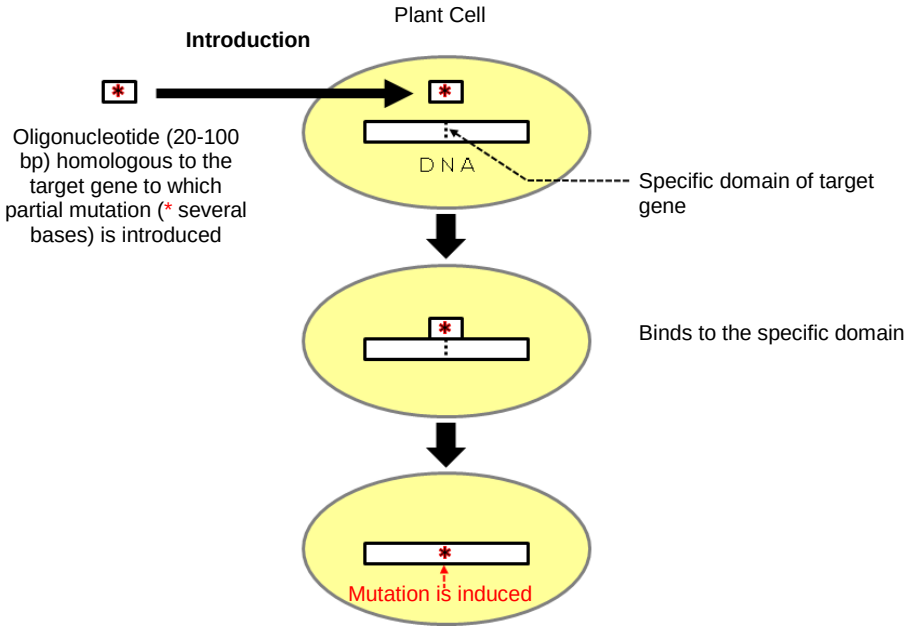

Fig. 3. Outline of ODM.

In ODM, mutation of about 1 base is induced to the target sequence on the genome, resulting in a change in the pattern for expressing the relevant gene. Such a mutation can occur naturally, and the mutation-induced crop cannot be distinguished from those selected through natural diversity or those developed by the conventional breeding techniques.

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[11] Oligonucleotide-Directed Mutagenesis
[12] A short sequence of DNA or RNA of around 20-100 base pairs

Using ODM, canola, maize, etc. with herbicide resistance have been developed to date. However, its low mutation rate is the bottleneck of its practical application.

(3) Cisgenesis/intragenesis

Cisgenesis is a method to introduce a gene of the same or related compatible species (cis-gene) to crops using GM technologies. Its presupposition is no introduction of genes or DNA fragments from any other species (Ezura and Osawa, 2013; Holme *et al.*, 2013).

In the conventional breeding by crossing, attempts to introduce a specific gene (e.g., pest resistance gene) from a wild relatives often result in causing harmful effects to other useful traits (e.g., yield, quality) possessed by the cultivars, which requires backcrossing with the cultivars after the introduction process. This backcrossing has to be repeated many times and over a long term. Additionally there is a difficulty in backcrossing vegetative propagation crops such as fruit plants, potato and sugar cane. In such occasions, cisgenesis may be an effective method of breeding.

Intragenesis also uses the same or related species as the source of genes in a similar manner as cisgenesis. However, intragenesis is used to control the expression amount of a specific gene by partially modifying the promoter[13] or terminator[14] that constitutes the gene (Ezura and Osawa, 2013; Holme *et al.*, 2013) (Fig. 4).

[13] A part of gene to which an RNA polymerase binds and starts gene transcription
[14] A part of gene which indicates cessation of gene transcription

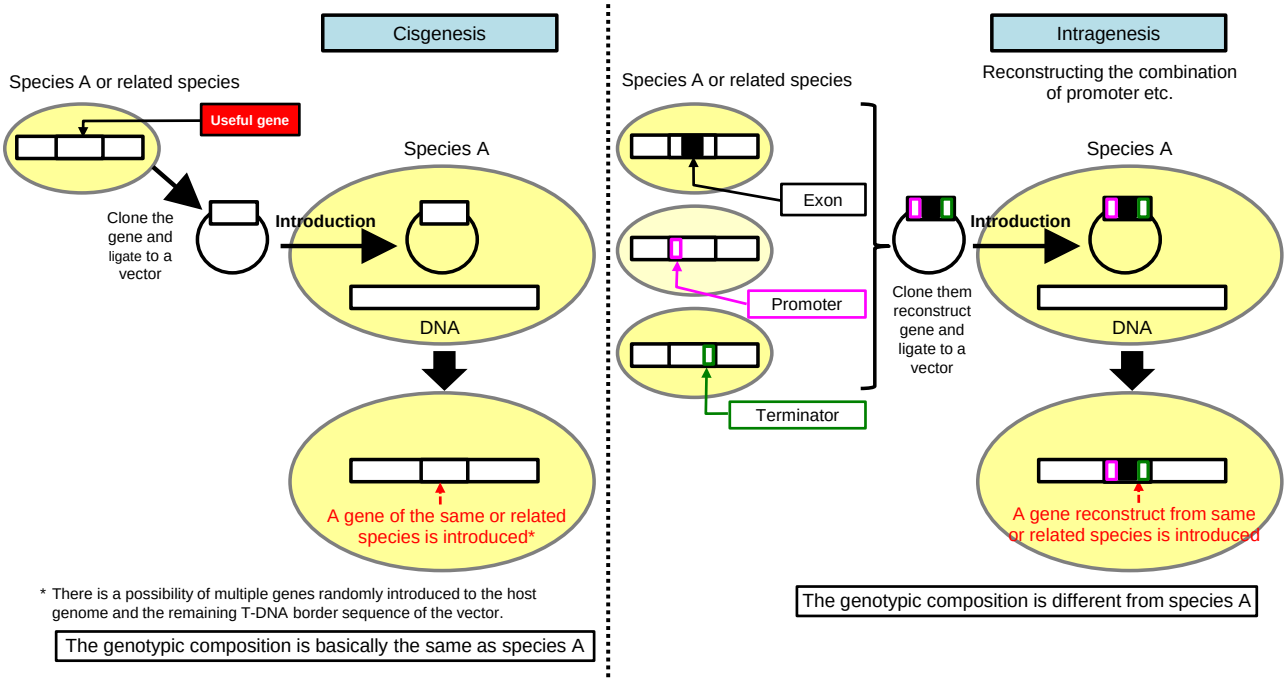

Fig. 4. Outline of cisgenesis and intragenesis.

In both techniques, cisgenes are introduced into plants by particle bombardment[15] etc. When the *Agrobacterium* method[16] is used, however, attention must be paid on the possibility of border sequences at both edges of vector's T-DNA domain (base sequence originating from *Agrobacterium*) being contained on the crop genome (European Food Safety Authority, 2012a).

(4) RNA-dependent DNA methylation

RNA-dependent DNA methylation (RdDM[17]) is a technique to methylate[18] some bases (cytosine) of domains involved with expression of specific endogenous gene (e.g., promoter) and thereby to control the expression of the relevant gene without altering the base sequence on crop genome (Ezura and Osawa, 2013).

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[15] http://www.nias.affrc.go.jp/gmogmo/FAQ/app/J6.html
[16] http://www.nias.affrc.go.jp/gmogmo/FAQ/app/J1.html
[17] RNA-dependent DNA methylation
[18] Refers to the addition of a methyl group to the C5 position of the pyrimidine ring of cytosine (C) constituting the promoter sequence domain. Expression of the gene is suppressed due to the methylation.

It is known that such a mechanism of RdDM is the same as a phenomenon that naturally takes place on a daily basis in the cells of organisms. For instance, in some varieties of morning glory the expressions of flower color synthesis genes vary depending on the domains of the petal, and some parts of petals exhibit irregular patterns (e.g., white blotches). It has become known recently that such a phenomenon is induced by RdDM (Suzuki, 2011).

To use RdDM in breeding, one shall construct a DNA fragment that is homologous to a part of the sequence of the promoter domain of the gene whose expression is to be suppressed (with its base sequence repeatedly sequenced in the opposite direction), and introduce the DNA fragment into a crop by using a vector.

In the cell of the crop, double-stranded RNA (dsRNA[19]) transcribed from the DNA fragment is produced, and the dsRNA is then decomposed into low-molecular-weight single-stranded RNAs (siRNAs[20]) through the biological reaction in the cell. It is said that the siRNA acts on the promoter domain of endogenous gene, inducing methylation of some bases (Pooggin, 2013; Chinnusamy *et al.*, 2009; O. Mathieu and Bender, 2004).

In RdDM, there may be two cases: the DNA fragment is transiently expressed in the cell without being integrated into the genome (disappears thereafter) and therefore is not to be inherited to progenies; and the DNA fragment is integrated into the genome and is to be inherited (Fig. 5).

[19] double stranded RNA
[20] small interfering RNA

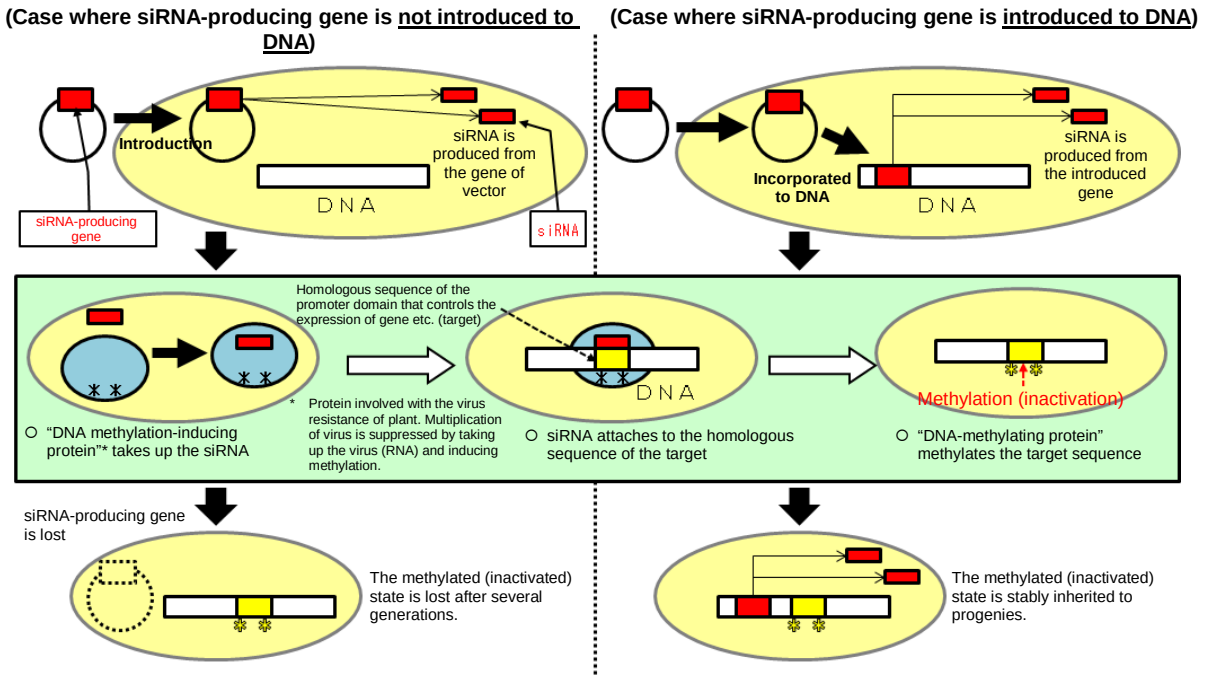

Fig. 5. Outline of RNA-dependent DNA methylation.

Even in the case where the DNA fragment is not inherited to progenies, the methylated state of the promoter is inherited for several generations at least. Therefore, it is possible to suppress the expression of specific endogenous gene without modifying the base sequence of the host genome.

(5) Grafting using GM rootstock

Grafting is a technique to artificially joint together a rootstock and a scion which are different plants from each other. Currently, grafting is generally used for fruit plants and vegetables (e.g., eggplant, tomato, cucumber).

The effects and purpose of grafting include 1) control tree vigor to make the fruiting age earlier or to improve yield, 2) dwarfing of trees to improve the efficiency of farm work, and 3) enabling of repeated cultivation by using a rootstock that is resistant to soil diseases. For instance, when a GM rootstock with resistance to a specific soil pest is developed and a scion of non-GM variety is grafted to the rootstock, it would become possible to avoid the effects of the soil pest without changing the quality etc. of the produce harvested from the scion (Koepke and Dhingra, 2013; Ezura and Osawa, 2013) (Fig. 6).

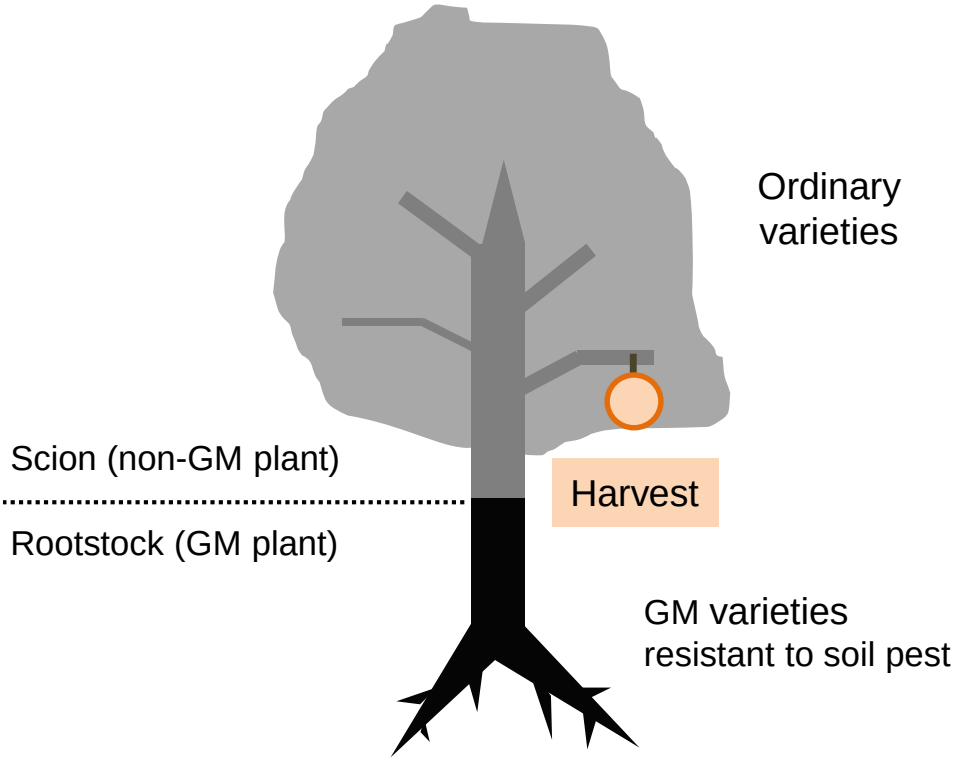

Fig. 6. Outline of grafting.

Generally, materials are transported from a rootstock to a scion through sieve tubes, and the transgenes (e.g., disease resistance gene) integrated into the genome of the rootstock do not migrate to the scion. However, it has become known recently that specific proteins or part of RNA produced in the rootstock are also propagated to the scion through the sieve tubes and control the strength, flowering time, etc. of the scion (Notaguchi *et al.*, 2008; Harada, 2010).

(6) Reverse breeding

Currently, F1 hybrid (F_1) is the most mainstream in breeding of maize and vegetables for the purpose of developing new varieties with traits such as high yield by taking advantage of the characteristics of heterosis[21].

F_1 plants are bred through the process of devoping the parental lines with pairing genetically-homologous chromosomes (homozygotes[22]), crossing them and selecting the descendent lines

[21] A phenomenon where the first crossed offspring exhibits superior traits in plant size, resistance to disease or the environment, etc. compared to the parental lines

[22] Those with exactly the same genes (base sequence) inherited from each parent on certain genetic loci

where the effects of heterosis is expressed (heterozygotes[23]). Reverse breeding is a technique to restore the parental lines (homozygotes) from the F_1 plants (heterozygotes) by retracing this process.

The specific procedure to restore the parental lines starts with obtaining a gamete (1n (pollen)) that is the same as the chromosome of the target parent lines (2n, homozygotes). First, in order to prevent recombination among chromatids during the meiosis period, a DNA fragment with a base sequence homologous to the endogenous gene involved with the relevant recombination (with the repeated sequence in the opposite direction) is developed and introduced into a crop.

Once introduced, double-stranded RNA (dsRNA) originating from the DNA fragment is produced inside the cell. Through RNA interference[24], expression of the endogenous gene involved with recombination during the meiosis period is interfered with, resulting in suppressed interchromosomal recombination. The thereby produced gamete (1n (pollen)) has exactly the same chromosomes originating from the parents. The parental lines (2n) could be restored by doubling of the chromosomes. (Ezura and Osawa, 2013; Dirks *et al.*, 2009; Matzke and Birchler, 2005) (Fig. 7).

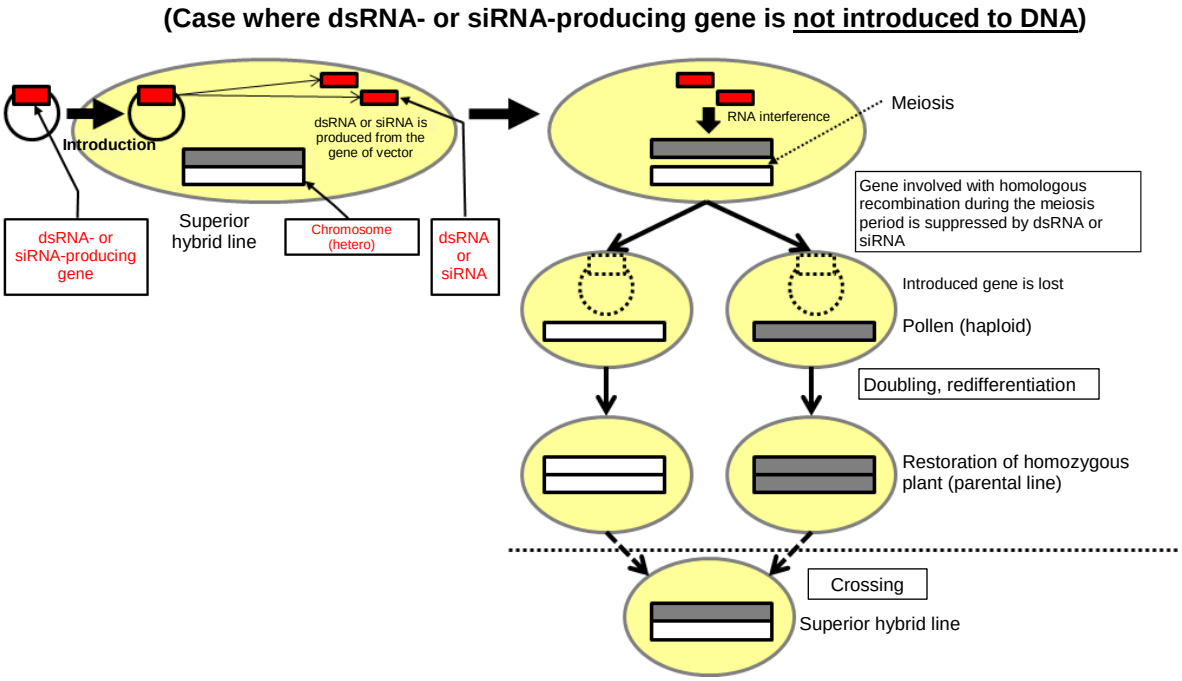

Fig. 7. Outline of reverse breeding.

[23] Those with different alleles on certain genetic loci inherited from parents

[24] A method to suppress the expression of a certain gene through artificial production of double-stranded RNA in the plant body by taking advantage of the phenomenon where mRNA with a base sequence complementary to double-stranded RNA gets decomposed

However, in the parental line group there are some plants where the DNA fragment introduced to suppress the interchromatid recombination still remains on their genome. Therefore, plants without the relevant DNA remaining must be selected by PCR etc. to be used as the parental line.

(7) Agro-infiltration

Agro-infiltration is a technique to locally infect a plant with *Agrobacterium* (bacteria) to which specific genes are incorporated, and to select plants with disease resistance, pest resistance, etc. by examining the expression of the relevant gene (level of the exhibited symptoms).

Agrobacterium is soil bacterium that infects the root or stem of beans and vegetables in nature and produces lumps called crown gall. Since *Agrobacterium* possesses the Ti plasmid[25] that can introduce DNA into the genome of plant, it is used as a vector (transporter) for developing GM crops.

When developing a GM crop, usually, cultured cells etc. are infected with *Agrobacterium* and the target gene is introduced into the genome of the plant through the Ti plasmid. In agro-infiltration process, however, only a particular organ of the plant body such as the leaf (limited to non-reproductive tissues) is infected. Therefore the target gene exists either in a free state in the cell without being integrated to the genome or, even if with being integrated to the genome, limited to the relevant organ (e.g., leaf) (Ezura and Osawa, 2013; Ohadi and Rasouli *et al.*, 2013).

By applying this technique, for instance when examining and selecting lines resistant to a specific viral disease from line groups obtained by normal crossbreeding, it becomes possible to estimate the existence of disease resistance by examining the disease symptoms (the level of the expression of the relevant protein) on the leaf etc. that are infected with *Agrobacterium*, in which a gene of the protein produced by the disease virus is incorporated (Fig. 8).

[25] A plasmid that is possessed by *Agrobacterium* and involved with the formulation of crown gall. It consists of the T-DNA domain that will be introduced into the chromosome of infected cells and the Vir domain that are involved with cutting out or incorporating the T-DNA domain into the host cell.

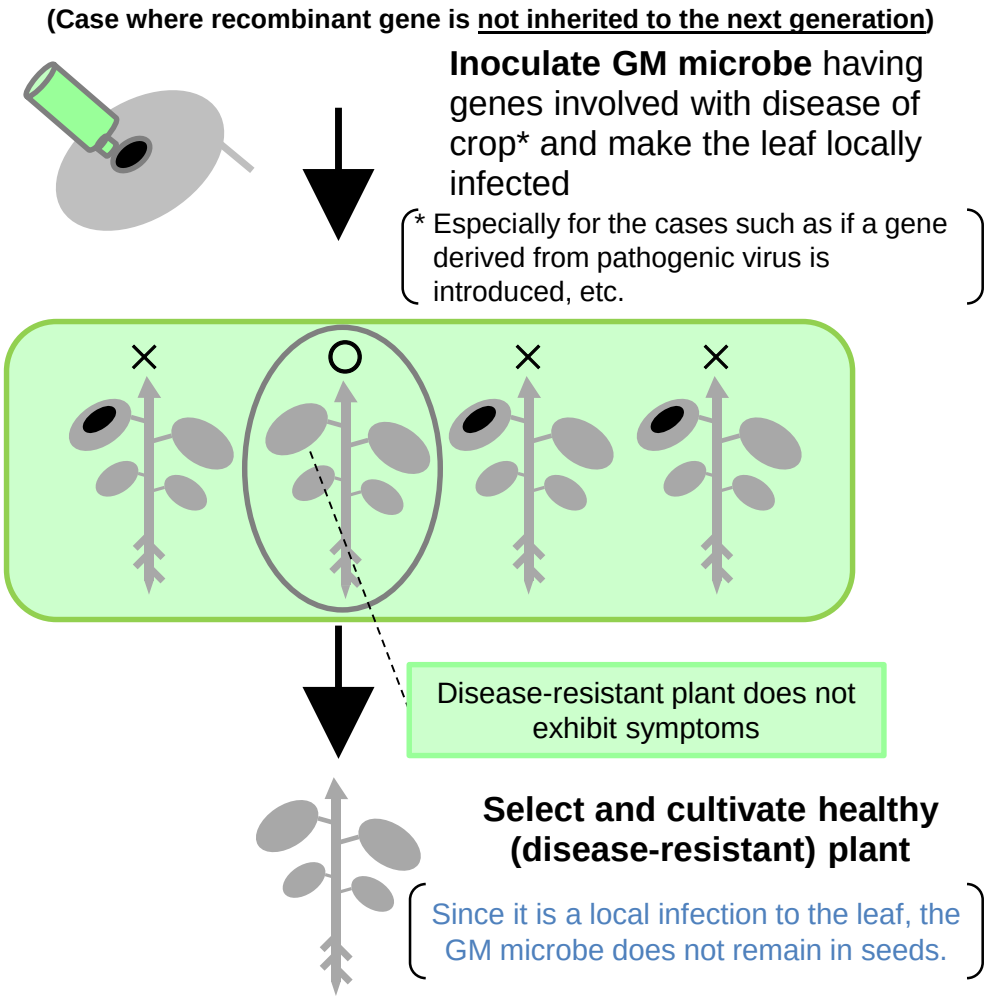

Fig. 8. Outline of agro-infiltration.

Infection with *Agrobacterium* is usually local and virus-like spreading of it throughout the plant is unlikely to occur. Therefore, it is considered that the genes originating from *Agrobacterium* do not remain in the body of a thereby examined and selected plant, as long as the infected domain (e.g., leaf) is removed.

As another method of agro-infiltration, there is a method targeting the reproductive tissues such as flowers. (floral dip). In this case, a transgene (aforementioned gene that codes the viral disease protein) is incorporated into the genome of reproductive cells, and the progenies are GMOs.

(8) Seed Production Technology (SPT) process

The SPT process is a technique developed by the US DuPont Pioneer to effectively produce F_1 hybrid maize seeds, and is already practically applied in the US.

Production of F_1 hybrid seeds of maize is generally carried out by crossing different parents. However, since one plant has both the tassel florets and ear florets, the tassel florets of female parents are usually cut (emasculation) to prevent inbreeding.

In order to omit the emasculation process, DuPont Pioneer developed a system that enables effective production of "SPT-maintainer lines" and "seeds for producing F_1 (non-GM)" by introducing a pollen sterility gene etc. into the line for producing F_1 seeds (homozygous). The SPT-maintainer lines have the recombinant genes heterozygously, and inbreeding the lines allows to efficiently produce seeds of the SPT-maintainer lines and the seeds for producing F1 which are male sterile (Fig. 9).

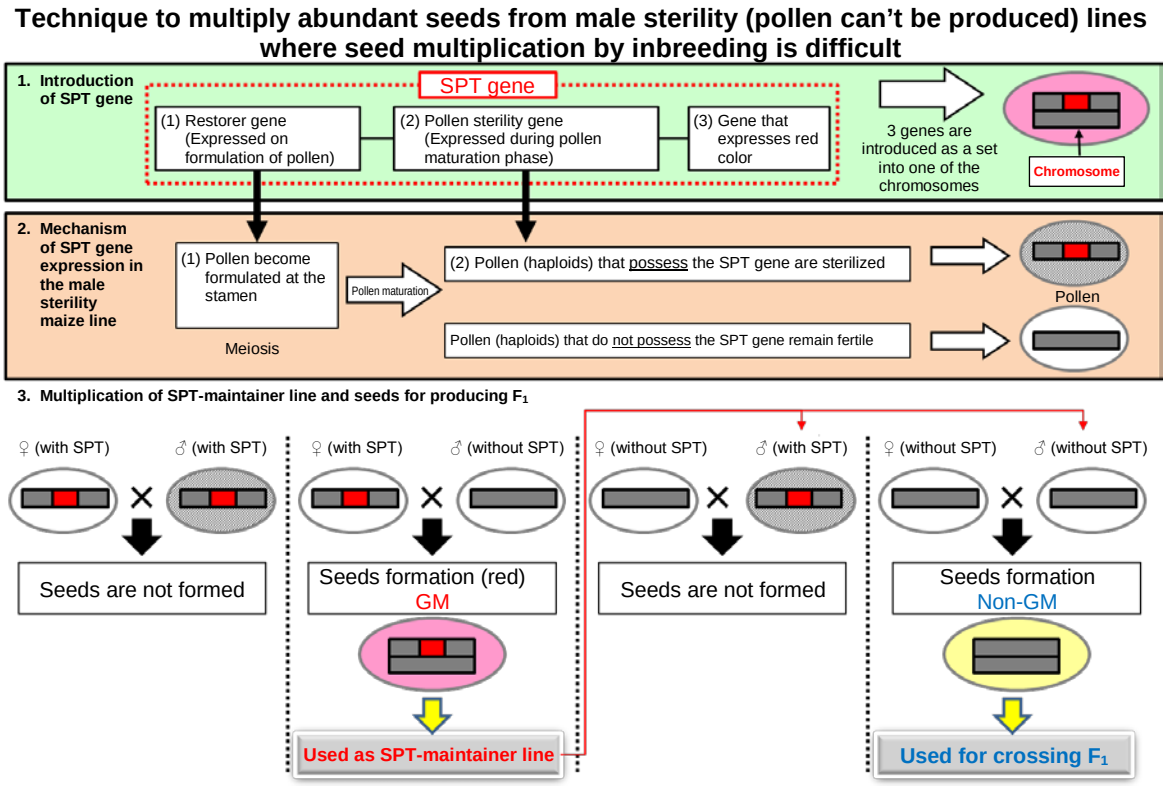

Fig. 9. Outline of SPT.

With the pollen sterility gene, a gene that expresses a red fluorescent protein in the grain is also introduced. Therefore, even if the seeds that possess those genes get mixed in the F_1 hybrid seeds, such grains with remaining transgenes can be identified by the color through a high-performance color sorting machine and removed[26].

[26] http://www.mhlw.go.jp/stf/shingi/2r9852000002tccm-att/2r9852000002tcwe.pdf

2. Status of Consideration on Regulatory Framework (abbreviation)

Regulatory framework for crops developed by NPBTs may vary country to country, depending on the range of organisms covered by the current GM regulations adopted by the country. Even if such crops may not be subject to GM regulations at the moment, the fact is that cutting-edge GM techniques are being used. Differences between countries in the concept on how carefully these crops shall be managed in the viewpoints of food safety and effects on the biological diversity may affect the future application of regulations on NPBTs and crops engineered by NPBTs.

From such viewpoints, mainly by scientist groups, overseas countries have summarized scientific opinions on the framework for NPBTs and their products under the GM regulations through examination of the characteristics of each breeding technique and comparison with conventional breeding techniques.

The study group has discussed with those information from overseas countries partially from URL listed as described below.

(1) EU

EASAC (European Academies Science Advisory Council) "Planting the Future"(2013)

http://www.easac.eu/fileadmin/Reports/Planting_the_Future/EASAC_Planting_the_Future_FULL_REPORT.pdf

European Commission "Directive 2001/18/EC" (2001)

http://www.biosafety.be/gb/dir.eur.gb/del.rel./2001_18/2001_18_tc.html

EFSA (European Food Safety Authority)

http://www.efsa.europa.eu/

EFSA "Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis" (2012)

http://www.efsa.europa.eu/en/efsajournal/pub/2561.htm

EFSA "Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function" (2012)

http://www.efsa.europa.eu/en/efsajournal/pub/2943.htm

EPSO (European Plant Science Organisation) "Crop Genetic Improvement Technologies" (2015)

http://www.epsoweb.org/file/2038

Greenpeace "Open letter to the Commission on new genetic engineering methods" (2015)

http://www.greenpeace.org/eu-unit/en/Publications/2015/Open-letter-to-the-Commission-on-new-genetic-engineering-methods/

JRC (Jount Research Centre) "Workshop on comparative regulatory approaches for new plant breeding techniques" (2011)

http://ipts.jrc.ec.europa.eu/presentations/NPBT.cfm

North American Agricultural Biotechnology Council "New DNA-Editing Approaches: Methods, Applications and Policy for Agriculture" (2014)

http://nabc.cals.cornell.edu/Publications/Reports/pubs_reports_26.html

Official Journal of the European Union "Regulation (EC) No.1829/2003 of the European Parliament and of the Council of 22 September 2003 on Genetically Modified Food and Feed" (2003)

http://ec.europa.eu/food/food/animalnutrition/labelling/Reg_1829_2003_en.pdf

Official Journal of the European Union "Directive 2009/41/EC of the European Parliament and of the Council" (2009)

http://eur-lex.europa.eu/LexUriServ/ LexUriServ.do?uri=OJ:L:2009:125:0075:0097:EN:PDF

(2) USA

Cibus

http://www.cibus.com/

EPA (Environmental Protect Agency)

http://www.epa.gov/

FDA (Food and Drug Administration)

http://www.fda.gov/

FDA "Food from Genetically Engineered Plants"

http://www.fda.gov/Food/FoodScienceResearch/GEPlants/default.htm

Health Canada "Novel Food Information – Cibus Canola Event 5715 (Imidazolinone and Sulfonylurea Herbicide Tolerant)"

http://www.hc-sc.gc.ca/fn-an/gmf-agm/appro/canola-5715-eng.php

USDA (United States Department of Agriculture)

http://www.aphis.usda.gov/

USDA "Biotecnology"

https://www.aphis.usda.gov/wps/portal/aphis/ourfocus/biotechnology

USDA APHIS (Animal and Plant Health Inspection Service) "Docket No. Aphis-2012-0067" (2014)

http://www.aphis.usda.gov/brs/fedregister/BRS_20141110b.pdf

White House "Executive Order 13563; Improving Regulation and Regulatory Review" (2011)

https://www.whitehouse.gov/sites/default/files/omb/inforeg/eo12866/eo13563_01182011.pdf

(3) Australia and New Zealand

FSANZ (Food Standards Australia New Zealand)

http://www.foodstandards.gov.au/

FSANZ "New plant breeding techniques workshops" (2012, 2013)

http://www.foodstandards.gov.au/consumer/gmfood/Pages/New-plant-breeding-techniques-in-the-spotlight.aspx

OGTR (Office of the Gene Technology Regulator)

http://www.ogtr.gov.au/

Scion

http://www.scionresearch.com/

(4) Other international trends

APEC (Asia-Pacific Economic Cooperation) "2010 APEC Ministerial Meeting on Food Security" (2010)

http://www.apec.org/Meeting-Papers/Ministerial-Statements/Food-Security/2010_food.aspx

OECD (Organisation for Economic Co-operation and Development) "Consensus Documents for the Work on Harmonisation of Regulatory Oversight in Biotechnology"

http://www.oecd.org/science/biotrack/consensusdocumentsfortheworkonharmonisationofregulatoryoversightinbiotechnology.htm

III. Examples of R&D in Japan and Considerations on the Effects on Biological Diversity

During the advancing R&D and initiatives towards practical application related to NPBTs mainly in the US and Europe, in Japan, such initiatives were taken only at a limited number of universities and research institutions. Full-fledged R&D on NPBTs in Japan had to wait for the announcement of the FY2013 commissioned project of MAFF, "Project to Develop the Next-Generation Production Basic Technologies of Agricultural and Livestock Products using Genomic Information" [27] (hereinafter referred to as "NG Genome Project").[28]

Therefore, the Study Group decided to summarize the general ways of thinking on the effect on biological diversity of NPBT, regarding three techniques promoted for practical use by MAFF in the project above as case studies.

Regarding the regulatory framework for NPBTs pertaining to food safety, the Study Group decided not to include it in the scope of its deliberations since that will be separately deliberated at the "Expert Committee on Genetically Modified Food" [29] established under the Newly Developed Food Investigating Group, Food Sanitation Subcommittee, Pharmaceutical Affairs and Food Sanitation Council, Ministry of Health, Labor and Welfare.

The Cartagena Act (Article 2, Paragraph 2[30]) defines a GMO subject to the regulation. One of them is an organism that possesses nucleic acid, or a replicated product thereof, obtained through use of a technology, as stipulated by the ordinance of the competent ministries, for the processing of nucleic acid extracellularly. Therefore, the Study Group decided to:

(1) deliberate the possibility of a foreign nucleic acid used for gene recombination or the replicated product thereof remaining in the plant; and,
(2) summarize the general ways of thinking of adverse effects on the biological diversity through the comparison with crops produced by conventional breeding techniques, or considerations on the possibility of unintended mutation occurring, etc., since there are rules to exclude organisms

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[27] http://www.s.affrc.go.jp/docs/project/information/h25/zisedai.htm
[28] Such R&D on NPBTs are transferred to the Strategic Innovation Promotion Program (SIP) of the Cabinet Office, where R&D is comprehensively and strategically progressed for a wider range of techniques and plants through the collaboration by the relevant ministries.
Reference URL: http://www8.cao.go.jp/cstp/gaiyo/sip/keikaku/9_nougyou.pdf
[29] http://www.mhlw.go.jp/stf/shingi/2r98520000008fcs.html#shingi148834
[30] http://www.bch.biodic.go.jp/english/law.html

that fall in the category of so-called “self-cloning” and “natural occurrence” from organisms subject to the regulations (Article 2, Paragraph 1 of the enforcement regulations of the Act[31]).

1. Techniques to Remove the Introduced Transgenes during the Breeding Process

(1) Accelerated breeding of fruit trees using early flowering genes

Seedlings of fruit trees such as citrus usually take 5-10 years to flower and fruit, which is a major obstacle to the development of new varieties. Especially when introducing useful traits (e.g., disease resistance) that exist in related wild species into commercial varieties, in order to remove unnecessary traits (defective traits) that exist in the related wild species to ensure the useful traits originally possessed by commercial varieties, backcrossing with commercial varieties must be repeated for many generations.

For instance, for apple (*Malus* × *domestica* Borkh.), there is a case where the apple scab[32] resistance gene (*Vf* gene) originating from a related varieties *Malus floribunda* 821 was introduced to the apple cultivar (variety name: ‘Goldrush’), and it is said that it took 7 generations over 68 years to achieve the desired result (‘Goldrush’) after crossing ‘Rome Beauty’ and *Malus floribunda* 821 in 1926.

In response to such a situation, in 1999, the *FT* (*Flowering locus T*) gene of thale-cress (*Arabidopsis thaliana*) was discovered by the group of Prof. Araki of Kyoto University, and it was confirmed in 2007 that the FT protein expressed by the gene is a plant hormone (florigen) that induces flower bud formation of plants (Corbesier *et al.*, 2007; Tamaki *et al.*, 2007). Applying this research result, currently, under the NG Genome Project, the National Agriculture and Food Research Organization, Institute of Fruit Tree Science is working on the development of accelerated breeding for citrus using this *FT* gene (Endo *et al.*, 2005; Endo *et al.*, 2009).

1) Development of accelerated breeding by introduction of *CiFT* gene

Citrus tristeza virus (CTV)[33] infects citrus such as Satsuma mandarin (*Citrus unshu*) and causes major damage, while it is known that CTV resistance gene exists in a related species, trifoliate orange (*Poncirus trifoliata*).

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[31] http://www.bch.biodic.go.jp/english/law.html
[32] Disease of apple, pear, etc. caused by some types of filamentous fungi where black spots emerge on the fruit or leaves
[33] Citrus tristeza virus

To introduce the resistance gene into citrus through cross breeding, crossing with trifoliate orange is to be made first, the hybrid seedling with CTV resistance and relatively good traits are to be selected, and selected plants are to be crossed with a citrus variety in order to remove the defective traits of trifoliate orange. This process needs to be repeated to achieve plants with the desired outcome.

However, assuming backcrossing needs to be performed for about 7 generations as was the case of the apple scab resistance gene, since it takes about 7-10 years (per generation) for hybrid seedlings to reliably flower and fruit, introgression of CTV resistance gene would require over half a century (7 generations × 7-10 years).

To that end, in this project research, *CiFT* gene[34], a homologue[35] of the *FT* gene, was introduced into trifoliate orange by the *Agrobacterium* method (Endo *et al.*, 2005; Endo *et al.*, 2009), which was then crossed with hyuganatsu (*Citrus tamurana*;cultivars). The hybrid seedling successfully flowered and fruited about 2 years after planting.

If the hybrid seedling is repeatedly crossed with hyuganatsu (a commercial variety) about 7 times, a commercial variety resembling hyuganatsu with resistance to CTV will be obtained in about 10 years according to the estimation. Additionally, during the process of selecting the hybrids, use of DNA markers that widely cover the genome of commercial varieties may further improve the efficiency of the selection, and it is prospected that the breeding period would be eventually reduced to about 5-7 years. Plants having *CTV* resistance gene without having the *CiFT* gene will segregate in the hybrid progenies; hyuganatsu plants resistant to CTV free from transgene (*CiFT* gene) will be obtained by selection using the DNA marker assisted selection.

2) Accelerated breeding using Apple Latent Spherical Virus (ALSV) vector

Even when a virus infects a plant, the virus RNA itself is not usually incorporated into the plant genome. Therefore, when a plant is infected by the virus with incorporated *FT* gene, the FT protein can induce early flowering in the plant without the *FT* gene incorporated into the plant genome.

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[34] *Citrus Flowering locus T*
[35] Gene that is considered homologous

Prof. Yoshikawa of Iwate University is developing a flowering promotion technique for apple by using one of the latent viruses, “Apple Latent Spherical Virus (ALSV[36])”.

Specifically, it was confirmed that when cotyledon of apple seedling (during the germinating period) was inoculated and infected with an *FT* gene-incorporated ALSV (FT-ALSV) through particle bombardment, FT protein was expressed during the seedling stage of apple, and that flower buds were initiated and bloomed in about 2 months (7-8 leaf stage). It was demonstrated that cross-pollinated seeds of apple can be obtained in 1 year by pollinating the thereby obtained pollen to the normal pistil of apple flowers. Since ALSV rarely infects pollen or seeds, the recombinant virus will not be found in the next generation apple seedlings. (Yoshikawa, 2012; Suzuki, 2011)

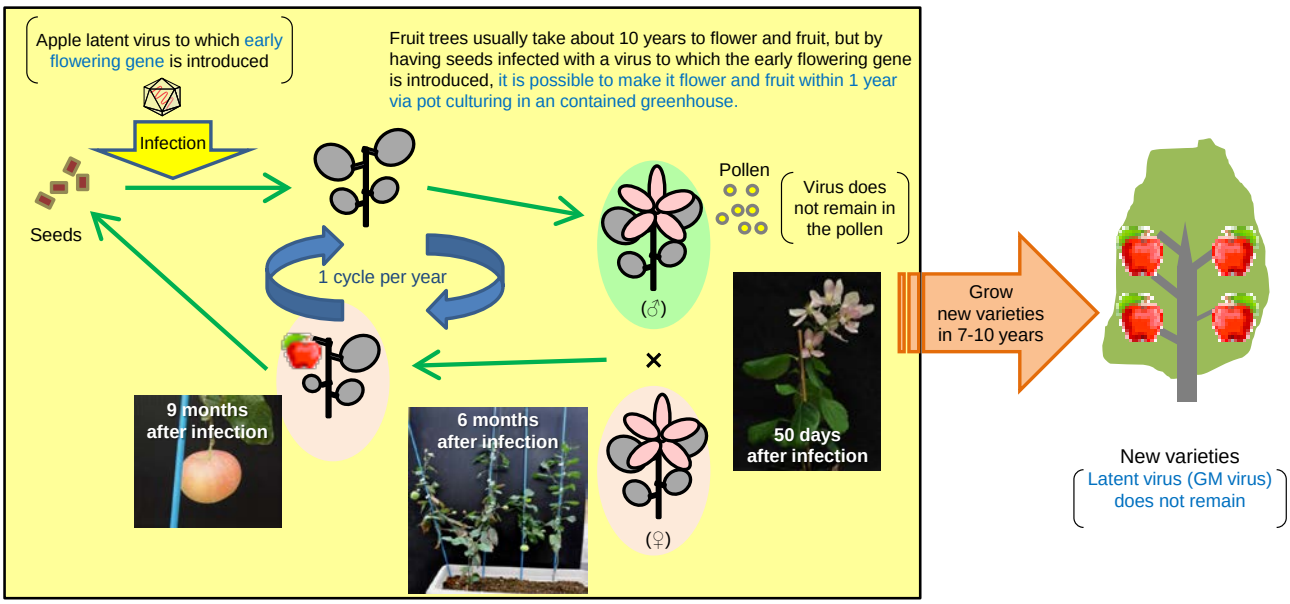

Fig. 11. Outline of applying flowering promotion technique by FT-ALSV.

In the experiment above, the flowering rate of seedlings was about 30%, and flowering occurred only once. In order to improve the flowering properties, a technique (RNA interference) to suppress the expression of flowering suppressor endogenous gene of apple is also currently in progress using separately developed ALSV that is incorporated with a flowering suppressor gene (*Terminal flower 1 (TFL1)* gene) (Sasaki *et al.*, 2011; Igarashi *et al.*, 2009).

When apple seedlings were infected with ALSV to which both the *FT* and *TFL 1* genes are incorporated, apple trees flowered at a rate of more than 90% and exhibited the characteristic of ever-flowering, meaning it has become possible to collect pollen throughout the year.

[36] Apple Latent Spherical Virus

This technique is applicable to various crops including other fruits, soybean and vegetables. Development of accelerated breeding for grapes, pears, etc. is currently in progress at Iwate University etc. under the Strategic Innovation Promotion Program (SIP) of the Cabinet Office, aiming at practical application with one crossing generation being reduced to one year or less.

(2) Recurrent selection of inbreeding crops such as rice

Usually, breeding by crossing two varieties is used for inbreeding crops[37] like rice. However, this technique has a limitation in the number of usable breeding material (genetic resources), and incorporation of various genes like those related to the yield ability (genes involved with the number of spikelet, photosynthetic ability, biomass amount, etc.) requires repeated crossing with various breeding material. As a result, development of ultra-high yielding varieties etc. in the future is considered to take a substantial amount of time.

Amid such a situation, taking into account the fact that the unit crop of maize (outbreeding crop[38]) has been steadily increased through breeding and improving over years, the National Agriculture and Food Research Organization, Institute of Crop Science (NICS) and the National Institute of Agrobiological Sciences (NIAS) undertook the development of a breeding system for applying the recurrent selection, which is generally used for breeding maize nowadays, to inbreeding crops such as rice to break through the limitation in their unit crop.

[37] Refers to plants where fertilization mainly occurs between the pollen and the egg cell of the same flower (or between the male flower and the female flower of the same plant for unisexual flowers); examples are rice, wheat, and soybeans

[38] Refers to plants where fertilization mainly occurs between flowers from different plants; examples are maize and buckwheat

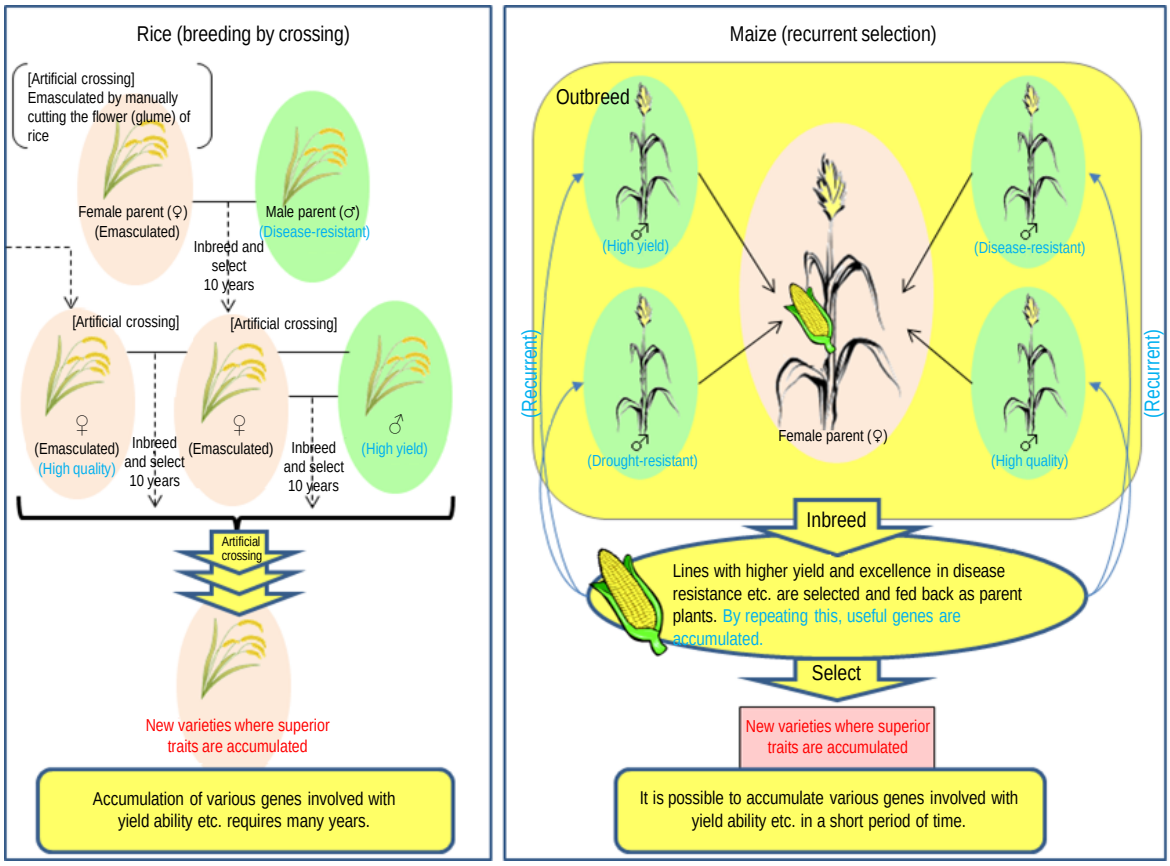

Fig. 12. Difference between the breeding technique for maize and that for rice.

Recurrent selection of maize is a method to accumulate genes that exist in maize by repeatedly crossing and inbreeding various breeding materials with different genetic backgrounds and thereby to breed an ideal parental group for producing hybrid with accumulated genes involved with yield, quality, environmental stress resistance, disease/pest resistance, etc. (Fig. 12).

At NICS and NIAS, a dominant male sterility gene etc. for making rice efficiently outbreed is first introduced to rice, and plants that have those genes heterozygously (trait transformants) are selected. As the pollen of the transformants becomes sterilized (i.e. outbreeding), by cultivating various breeding materials with different genetic backgrounds around them, outbred plants (F_1) are automatically obtained. About half of the progenies obtained inherit the male sterility trait (Fig. 14).

As a herbicide resistance gene (positive trait gene) and a conditional lethal (negative market gene) are individually incorporated into the cassette of the male sterility gene cassette introduced to the relevant rice (Tanaka, 2010) (Fig. 13), among the progenies:

(1) positive selection (e.g., herbicide treatment) results in selecting about half of the survived rice that inherited the male sterility trait; and,

(2) negative selection results in selecting plants without possessing the transgenes of the male sterility gene etc. (about half of all plants).

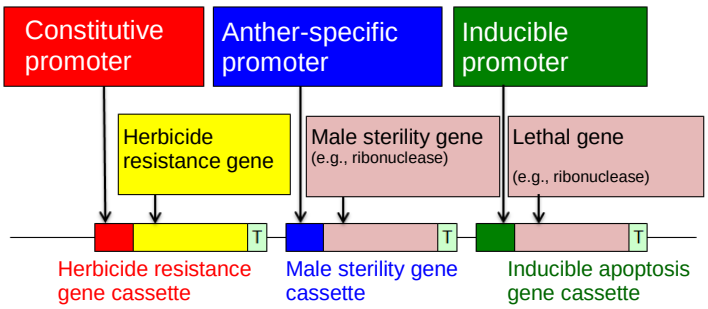

Fig. 13. Schematic illustration of introduced gene cassette.

New transgene free varieties with superior yield are to be produced by repeating such selection as crossing and fixation of the important traits in the negative selection (Tanaka, 2010).

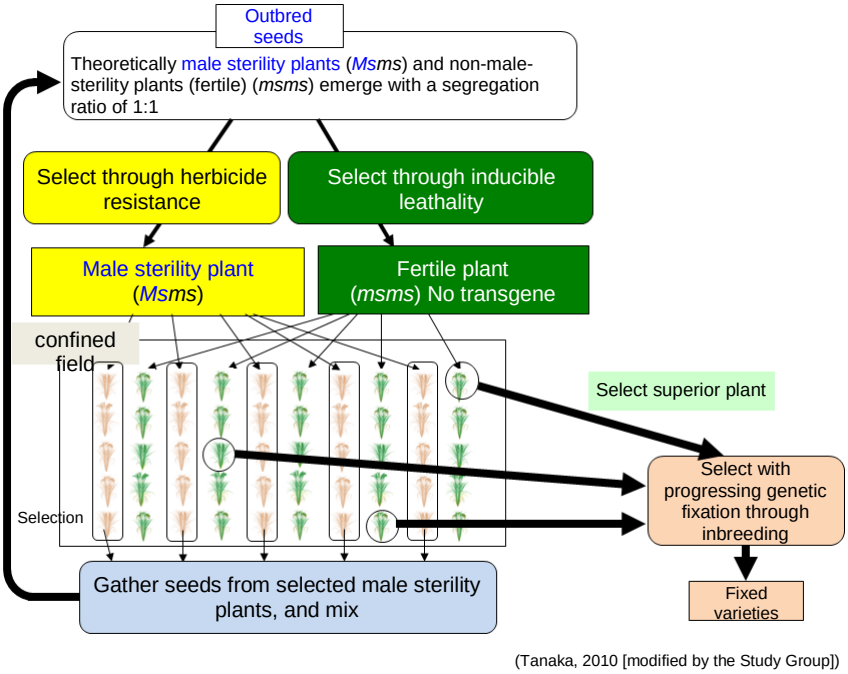

Fig. 14. Outline of recurrent selection for inbreeding crops such as rice.

NICS and NIAS are planning to establish such a breeding system in the next 5 years or so and to produce and supply new promising varieties (lines) one after another.

(3) Considerations on the effects on biological diversity

1) Possibility of transgenes used for recombination or their copies remaining

For either technique, at the initial stage of breeding, plants, in which transgenes (e.g., *CiFT* gene, *TFL 1* gene, pollen sterility gene) or recombinant viruses (ALSV) remain, emerge.

Therefore, during the initial stage of breeding where crossing and selection are carried out (experimental research stage), appropriate use in accordance with the existing Cartagena Act (e.g., application for the approval to use in a closed system or to test at an confined field) is required.

Meanwhile, since the final commercial product can be selected from those with no relevant transgenes remaining (Null Segregant), it may be exempted from the regulations of the existing Cartagena Act if the absence of transgenes is confirmed by an appropriate method including PCR, Southern hybridization, and the next-generation sequencer.

Therefore, it is necessary for developers to provide the regulatory authorities with the detailed information (e.g., what kind of crossing and selection processes have been used to breed the final commercialized products) in advance and to receive judgment on the applicability of regulations to their future products.

2) Comparison with conventional breeding and possibility of unintended mutagenesis

Transgenes and recombinant viruses used for the breeding techniques above are used to improve the efficiency of breeding in crossing or selection process, and it is essential to confirm that the relevant transgenes do not remain in the final commercialized varieties. Then, such newly developed crops (new varieties) can be regarded as those produced by conventional breeding techniques.

Additionally, in breeding of crop plants, usually, there is a process to eliminate undesirable plants (plants with defective traits) from the line groups engineered. As a result, only the plants with good qualities and ease of growth become the selected varieties.

From the above understanding, if it is confirmed that transgenes are not included in the final crop, the crop can be regarded the same as those produced by conventional breeding such as crossing. Therefore, the Study Group considered that there are no matters that call for specific concerns over the effects on biological diversity.

2. Genome Editing using Artificial Restriction Enzymes

(1) Outline of R&D

Regarding SDN-1 that is on the extension line of conventional mutation breeding, while it allows for destruction of the action of genes by causing deletion of several bases after cleaving the target genes, it is not adequate for creating mutants where the base sequence on the genome is altered only for one base (Single Nucleotide Polymorphism (SNP[39])).

To that end, NIAS is promoting the development of techniques to accurately induce mutation to specific genes of crop genome for one to several bases only (point mutation) using SDN-2.

Additionally, currently, effective expression of artificial restriction enzymes in plant bodies unavoidably requires incorporating artificial restriction enzyme genes (transgenes) onto the plant genes. Therefore, NIAS is also working on the development of a technique to completely remove transgenes by taking advantage of piggyBac, one of the transposons[40] of insects, after mutation (deletion, substitution or insertion for one to several bases) is induced to target genes.

By applying these techniques to breed crops, it will become possible in the future to suppress genes that generate allergens in rice or those that generate toxic substances in potato (e.g., glycoalkaloids) and to develop varieties with valuable novel phenotypes in a very short period of time, such as feed rice that is rich in a specific amino acid, sweeter crop that is rich in oligosaccharide, tomato with higher rate of flowering and higher yield, parthenocarpic tomato and capsicum that ripen even under low temperature in winter, grass that is easily digested by livestock, and pollen-free Japanese cedar.

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[39] Single Nucleotide Polymorphism
[40] DNA (base sequence) whose location on the genome is transportable within a cell

(2) Considerations on the effects on biological diversity

1) Possibility of transgenes used for recombination or their copies remaining

For both SDN-1 and SDN-2, usually, an artificial restriction enzyme gene (transgene) will be incorporated into the plant genome by means of *Agrobacterium* etc. Therefore, until the transgene is completely removed by carrying out, for instance, backcrossing with non-GM variety afterward, appropriate use in accordance with the existing Cartagena Act shall be required.

In SDN-2, a short guiding DNA fragment is introduced into a cell at the time of DNA repair after cleavage and the repaired sequence on the genome is artificially controlled. As a result a base sequence that is the same as the guiding DNA fragment is inserted into the produced crop. Therefore, these crops may be regarded as GMOs subject to the regulations of the Cartagena Act.

However, SDN-2 is to induce artificial substitution or insertion to a single or several-base nucleic acid, and a similar mutant may also be produced by SDN-1 albeit low in possibility. Additionally, a similar level of deletion, substitution or insertion of bases can occur in selection from natural diversity, conventional mutation breeding, etc.

Furthermore, in the existing Cartagena Act, even GM techniques have been used to develop them, the organisms where the same substitution of nucleic acid can occur under natural conditions (so-called “natural occurrence”) can be exempted from its regulations.

Therefore, there is a possibility that organisms produced by SDN-1 can be exempted from the existing Cartagena Act regulations. For SDN-2, it is important for the R&D side to actively gather and analyze related information (e.g., characteristics of mutated trait and whether the same mutants can be produced by other techniques), submit them to the regulatory authorities and ask for judgment on the applicability of regulations on a case-by-case basis.

For proving that the artificial restriction enzyme genes (relevant transgenes) are surely removed, it is required to carry out analysis using PCR, Southern hybridization, next-generation sequencer, etc. and to receive confirmation by the regulatory authorities.

2) Comparison with conventional breeding and possibility of unintended mutagenesis

While both SDN-1 and SDN-2 induce deletion, substitution or insertion of bases to the specific domain of crop genome, such mutation for several bases occurs in selection from natural diversity

or via conventional breeding by crossing, mutation breeding, etc. in an unintended form (Abe *et al.*, 2013).

Additionally, it is considered that the chance of unintended mutation occurring is lower, and that the risk on biological diversity etc. is reduced more than conventional mutation breeding by radiation, chemicals, etc. causing random mutation on multiple locations on the genome since SDN-1 and SDN-2 allow arbitrary modification of the target genes only.

Scientific knowledge on base deletion, substitution or insertion that can occur naturally or by conventional breeding techniques is still limited, therefore gathering and accumulation of more knowledge and findings are required. However, at least for SDN-1 and SDN-2 where mutations are induced at several-base levels, such mutations are considered to occur by conventional mutation breeding as well.

Further, as described in II-1-(1) (page 7), the possibility of off-target (which triggers unintended mutation) is expected to be extremely low for both techniques, and even if unintended mutation occurred to traits other than the intended trait by accidentally cleaving the domain other than the target gene, it is also expected that such plants are usually removed through the selection process of breeding.

From the above understanding, regarding SDN-1 and SDN-2 that induce mutation to several bases, if it is confirmed that no artificial restriction enzyme (transgene) is included in the final products, the products can be regarded the same as those produced by conventional mutation breeding. Therefore, the Study Group considered that there are no specific concerns over the effects on biological diversity.

However, at this stage, it is expected that there may be cases where sufficient knowledge is not available on the characteristics of mutated target genes, the expression mechanism of the intended trait, etc. In such cases, it is appropriate for developers to provide the regulatory authorities with the related information (e.g., detailed information on the breeding process, characteristics of the target mutated gene in the crop, introduced trait etc.) in advance so that the risk pertaining to the effects on biological diversity can be appropriately predicted, and to receive scientific assessment from experts as necessary.

IV. Points to Be Considered toward Future R&D and Practical Application

1. Promotion of Related R&D

In the situation of advancing globalization of agricultural product trading, the urgent task to radically strengthen the international competitiveness of Japan's agriculture is the development of epoch-making agricultural technologies that will bring breakthroughs to the agriculture in Japan.

Especially, in the field of agricultural breeding, epoch-making grain varieties etc. are developed one after another by utilizing GM techniques based on the US, and the cost reduction by reducing the amount of agrochemicals used and the increase of yield have been achieved. On the contrary, in Japan, along with the increasing difficulty in cultivating GMOs domestically, the options of techniques usable for breeding main crops including rice are limited, and it takes many years to develop new varieties.

Regarding vegetables and flowering plants, for which personal breeding is actively performed, Japan's high quality products are highly valued overseas, and the export of seedlings has been steadily increasing in recent years, especially to Asia. However, it has become difficult to obtain useful plant genetic resources from overseas as breeding materials, and there is a concern over stagnancy spreading in the development of new varieties.

NPBTs are techniques that basically draw out the maximum potential of agriculturally useful genetic traits that exist in the same or related species, which may efficiently improve the quality, functionality, yield, etc. of crops, and thereby NPBTs enable creation of unprecedented epoch-making varieties in a short period of time. In case the crops engineered by NPBTs do not possess transgenes at the end and have the same gene composition as crops produced by conventional breeding, risks on the food/feed safety and the effects on biological diversity can be suppressed. Additionally, NPBTs may allow for reduction of development cost involved with the compliance with GM regulations, so there is a possibility for Japanese private companies etc. to apply NPBTs to breeding and improving various crops including vegetables and flowering plants.

To that end, while paying attention to the trend of rapidly advancing R&D in the US and Europe, Japan should consider NPBTs as part of its next-generation breeding techniques and make strategic investment into R&D, from basic R&D to applied R&D.

Especially, the "Comprehensive Strategy on Science, Technology and Innovation 2015[41]" decided by the Cabinet in June 2015, indicates the development of next-generation breeding systems such as NPBTs as one of the priority R&D initiatives to be promoted in Japan, and the relevant ministries are jointly promoting R&D under the Strategic Innovation Promotion Program (SIP) of the Cabinet Office. Under the SIP, the government (research institutions under MAFF, MEXT, etc.), academia (universities) and industry (related private enterprises such as those in the breeding industry) shall collaborate in actively implementing initiatives in the development of Japanese NPBTs, conversing them into intellectual properties, creating innovative varieties of various plants including vegetables and flowers as well as grains through application of NPBTs, enhancing regulatory science, promoting direct dialogue with citizens for developing science communication, etc.

2. Initiatives towards Promoting the Social Understanding

(1) Compliance with GM regulations

In NPBTs, GM techniques are used and crops introduced with transgenes are handled during certain points of the breeding process. Therefore, appropriate handling in accordance with the existing Cartagena Act[42] is required during the breeding process.

With regard to cultivating the resultant novel engineered varieties or using them for food or feed, prior consultations with the regulatory authorities is necessary since there remains the possibility that GM regulations in accordance with the Cartagena Act[43], Food Sanitation Act[44], or Feed Safety Act[45] apply respectively.

On making judgment on the applicability of such GM regulations, the judgement criteria may be (1) whether the transgene used for recombination remains, and (2) whether the same crop can be selected from natural diversity or developed by conventional breeding techniques. Therefore, it is necessary for developers to actively provide the regulatory authorities with the related information (e.g., detailed information on the breeding process, characteristics of the target mutated

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41 http://www8.cao.go.jp/cstp/sogosenryaku/2015.html
42 http://www.lifescience.mext.go.jp/bioethics/anzen.html#kumikae
43 http://www.maff.go.jp/j/syouan/nouan/carta/about/sop_eng.html
44 http://www.mhlw.go.jp/stf/seisakunitsuite/bunya/kenkou_iryou/shokuhin/idenshi/index.html
45 MAFF website: http://www.maff.go.jp/j/syouan/tikusui/siryo/index.html
Food and Agricultural Materials Inspection Center website: http://www.famic.go.jp/ffis/feed/sub1_tuti.html

genes in the crop, introduced trait etc.) so that the risk pertaining to food and feed safety and the effects on biological diversity can be appropriately predicted. It is also necessary to assuredly and thoroughly conduct prior consultations. Such activities will become the first step towards fostering the social understanding of NPBTs.

(2) Methods to provide public with information and to communicate with it

Since many NPBTs use the latest molecular biological findings, in order to socially implement such cutting-edge techniques, it is important to promote interactive communications with various interested parties from the R&D stages, provide explanations on the meaning, contents, etc. of R&D in an easy-to-understand manner, and to reflect the voices of anticipation, anxiety and concerns from such parties on the R&D and the process of practical application. Additionally, considering the lingering social anxiety over the agricultural products and food utilizing GM techniques, the key point will be how to successfully disseminate information and communicate in a convincing manner that, (1) development of crops that are resistant to various environmental stresses and are superior in yield is an urgent task to address the issues including the global environmental changes and food shortage, and use of NPBTs that drastically increase the speed of crop breeding is extremely important, (2) similar plants can be selected from natural diversity or developed by conventional breeding techniques, etc., along with showing the actually developed crop plants (new varieties), while promoting the development of epoch-making novel varieties that bring tangible benefits to the farmers and consumers in Japan.

To that end, it is important to continue to collect related scientific findings and formulate opinions about the effects on biological diversity etc. at scientific panels like the Study Group, and foster the sense of trust in the nation on the use of NPBTs through promoted communications with a broader range of knowledgeable persons, consumer groups, mass media, producers, industry, etc. based on such scientific opinions, establishing easy – to understand guidance.

(3) Promotion of international harmonization pertaining to regulatory framework

Currently each country or region is considering the regulatory framework for NPBTs, and there is a possibility that differences among the frameworks could cause confusion in the trade of agricultural products in the future. More specifically, Japan imports a large amount of crops from the US and other countries, and it will be inevitable to coordinate and have discussions with trading partners on the regulatory framework.

It will be important to accelerate the formulation of scientific opinions etc. in Japan and to contribute to sharing such scientific information and views, and thereby international harmonization pertaining to regulatory framework for NPBTs at suited opportunities such as the OECD-WG in the future.

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Terminology

Agrobacterium method

A gene introduction method taking advantage of the principle of soil bacterium *Agrobacterium* incorporating its genes into plants in nature.

Specifically, this technique uses the characteristic of *Agrobacterium* incorporating a part (section called "T-DNA") of transporter DNA (plasmid) into plant cells. A target gene can be introduced to the chromosome (genome) of plant cells by removing the virulence gene, incorporating a gene involved with an agriculturally useful trait there, and inserting it into a plant cell.

CRISPR/Cas system

CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats. It is an artificial restriction enzyme technique using the acquired immune mechanisms for phages or plasmids of prokaryotes (e.g., bacteria) where the system to cleave and eliminate extraneous DNA is applied.

Different from ZFN or TALEN, the CRISPR/Cas system identifies the base sequence information on the genome (DNA binding domain) by RNA instead of protein, and designing of the artificial restriction enzyme is easier than ZFN or TALEN.

Breeding by crossing

A breeding technique to cross genetically different varieties to develop crossbreed groups with various mutations and to select and grow plants with superior traits among them. It is a common technique used for breeding various crops including rice.

Southern hybridization

A technique to detect DNA, which was devised by Edwin Southern.

DNA fragments with various lengths cut by restriction enzymes are put through agarose gel electrophoresis, and the existence of the target DNA is defined by the positions of DNA fragments segregated on the gel.

Next-generation sequencer

A method to determine a substantial amount of base sequences by simultaneously analyzing millions of randomly cut DNA fragments. It enables a wide range of analysis including whole genome sequence, epigenome analysis (e.g., analysis of DNA methylation state) and transcriptome analysis (analysis of RNA as transcript).

DNA marker assisted selection

As described in Page 1, a breeding technique to use the base sequence information near the specific gene involved with an agriculturally useful trait as the marker (DNA marker) and to efficiently select plants that possess that base sequence.

For instance, breeding and selection of new varieties superior in disease resistance by conventional breeding methods can be achieved only by cultivating a large amount of crossbred plants at experimental fields and by investigating the disease symptoms. However, by using DNA markers, disease-resistant plants can be selected in a short period of time only by extracting and analyzing DNA of leaf etc.

Mutation breeding

A breeding technique to artificially induce mutation to the gene on plant genome by irradiation, chemical treatment, etc. and to select crops with superior agricultural traits from such plants.

For instance, in Tottori Prefecture which is a major producer of pears, a variety named "Nijisseiki" has been widely grown, but this variety is weak against black spot, and the disease prevention cost has long been a major issue. Then, a new varieties named "Gold Nijisseiki" which has black spot resistant is selected by NIAS (radiation breeding field), and Gold Nijisseiki now accounts for approximately 40% of pears grown in Tottori Prefecture.

Transcription Activator Like Effecter Nuclease (TALEN)

An artificial restriction enzyme technique where the infection mechanism of plant pathogen Xanthomonas is applied.

Similar to ZFN (Fig. 1 on Page 7), TALEN is an artificial restriction enzyme consisting of "DNA cleaving domain (*Fok* I)" with restriction enzyme activity and "DNA binding domain" (TALE) that identifies specific base sequence information on genome. At the DNA binding domain, while ZFN identifies the sequence by 3-base units, TALEN identifies by 1-base units.

Particle bombardment

One of the methods to introduce DNA into cells.

DNA is introduced by dusting fine particles of gold or tungsten with DNA and injecting them into cells by means of helium gas pressure etc. Generally, this method is used for plants with a low gene introduction rate by the *Agrobacterium* method (e.g., maize).

PCR method

PCR stands for Polymerase Chain Reaction. It is a method to exponentially multiply DNA by repeating the reaction to replicate a specific domain of DNA using two DNA fragments (primers) that sandwich the specific domain of DNA and enzyme that are involved with DNA replication

(polymerase). The PCR method is established as the basic technique of gene research for various purposes including determination of gene sequence and quantification of genes.

It has now become possible to confirm the existence of DNA (gene) even if the amount of target DNA contained in the analysis specimen is extremely small (about 10 copies).

List of Members of the New Plant Breeding Technique Study Group

Name	Position	Specialty
Ryo Ohsawa	Professor, Faculty of Life and Environmental Sciences, the University of Tsukuba	Plant breeding
Hiroshi Kamada*	Professor, Faculty of Life and Environmental Sciences, Gene Research Center, the University of Tsukuba	Molecular biology
Masakazu Shimada	Professor, Department of General Systems Studies, Graduate School of Arts and Sciences, the University of Tokyo	Conservation ecology
Masashi Tachikawa	Professor, Department of Regional and Environmental Science, College of Agriculture, Ibaraki University	International GMO policy
Masahiro Nakagawara	Vice Chairman of Working Group on Harmonisation of regulatory Oversight in Biotechnology, OECD	Plant breeding
Nobuyoshi Nakajima	Head of Ecological Genetics Research Section, Center for Environmental Biology and Ecosystem Studies, National Institute for Environmental Studies, Japan	Plant physiology
Akihiro Hino	Vice General Manager of the Central Laboratory, Nippon Flour Mills Co., Ltd.	Genetic biochemistry, GM detection technology

Note: Member Nakagawara was elected as the Chairman through mutual election

* The late Professor Hiroshi Kamada participated as a member until March 2014

History of Deliberations

○ 1st meeting: October 4, 2013 (Fri)
<Agenda>
(1) About the Trend surrounding NPBTs
(2) About the Immediate Directionality of Considerations
(3) Other

○ 2nd meeting: October 29, 2013 (Tue)
<Agenda>
(1) About the Operating Status of Self-Cloning and Natural Occurrence, etc.
(2) Case Study about NPBTs
(3) Other
<Details of Case Study>
(1) Fruit Plant Accelerated Breeding using Early Flowering Gene
(2) Application of Recurrent Selection to Inbreeding Crops such as Rice

○ 3rd meeting: November 27, 2013 (Wed)
<Agenda>
(1) Case Study about NPBTs
(2) About the Interim Report
(3) Other
<Details of Case Study>
Mutation Breeding using Artificial Restriction Enzyme etc.

○ 4th meeting: December 19, 2013 (Thu)
<Agenda>
(1) About the Overseas Trend Investigation Report
(2) About the Consideration on the Development and Practical Application of Crops using NPBTs (Interim Report)
(3) Other

○ 5th meeting: March 25, 2014 (Tue)
<Agenda>
(1) About the Results of OECD Working Group on the Harmonisation of Regulatory Oversight in Biotechnology and Workshops on NPBTs
(2) About the Consideration on the Development and Practical Application of Crops using NPBTs (Interim Report)
(3) About the Strategic Innovation Promotion Program (Cross-ministerial Strategic Innovation Promotion Program: SIP)

(4) Other
○ 6th meeting: March 27, 2015 (Wed)
<Agenda>
(1) About the Recent Trend in Japan and Overseas
(2) About the Study Group Report
(3) Other

○ 7th meeting: July 22, 2015 (Wed)
<Agenda>
(1) About the Report of APEC-HLPDAB
(2) About the Final Report (Draft)
(3) Other