Development and Evaluation of Comprehensive Detection Methods for Genetically Modified Crops

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and Sciences for Sustainable Development)

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Chapter 1

General introduction

1.1 Genetic recombination technique and genetically modified organisms

Since the discovery of the double helix structure of deoxyribonucleic acid (DNA) by James D. Watson and Francis H. Crick in 1953, there has been accelerated progress in molecular biology (Watson and Crick, 1953). A great deal of knowledge on DNA purification and the enzymes useful for DNA manipulation such as ligases and restriction enzymes has accumulated. The first genetic recombination in vitro was performed by Stanley N. Cohen and Herbert W. Boyer in 1973 (Cohen et al., 1973). Since then, many kinds of genetic recombination techniques have been developed and applied to extremely varied types of organisms. These techniques are now routinely used in laboratories for scientific research and genetically modified organisms (GMOs) are utilized for the production of food, feed and pharmaceuticals.

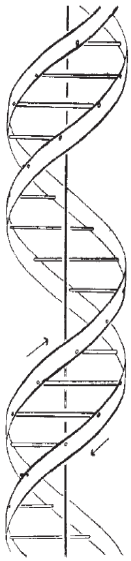

Fig. 1.1. DNA double helix structure. Two ribbons symbolize the two phosphates and sugar chains, and the horizontal rods the base pairs holding the chains together. The vertical line marks the fiber axis (source: Watson and Crick, 1953).

1.2 Genetically modified crops

Up until the era of the industrial revolution, the majority of human beings lived in an agrarian society and obtained many sources for life from plants. Over the centuries, human beings have cultivated and selected useful plants as agricultural crops and improved their traits, whether consciously or unconsciously, to suit their needs (Hino et al., 1999). Gregor Mendel discovered the basic theories of plant inheritance in 1865 (Mendel, 1865). However, Mendel's laws did not attract attention until 1900, when Hugo de Vries and Carl Correns rediscovered Mendel's work and Mendelian genetics became widely known. Practical crop breeding based on Mendelian inheritance laws was started and various breeding techniques, including selection breeding, cross breeding, back cross breeding, mutant breeding and polyploidy breeding, were developed. Currently, due to crop breeding, higher crop yields are consistently obtained. However, global population growth is continuing and the demand for food is expected to increase accordingly. Further crop improvement is thus needed to achieve much higher agricultural productivity for this increased population.

Crop improvement based on conventional breeding requires a long period of time, at least several generations of the plants. In the case of woody plants, it can take decades. Thus, scientists thought that genetic recombination would make crop improvement more systematic and quick. Additionally, transferring traits beyond the species barrier expands the potential of plant breeding. Many scientists have thus investigated plant genetic engineering. Concerning the gene transferring technique, *Agrobacterium tumefaciens* (now classified as *Rhizobium radiobacter*)-mediated gene transferring, electroporation and particle bombardment transformation were developed.

In 1994, the "Flavr Savr" tomato developed by Calgene Inc. became the first commercially available genetically modified (GM) plant (Hino et al., 1999). The GM tomato's polygalacturonase activity was suppressed by the antisense ribonucleic acid (RNA) technique under genetic transformation to keep the GM tomatoes firmer and fresher than

conventional tomatoes during transportation and in households. In the U.S. state of California, GM tomatoes were commercially cultivated on 10 hectares.

In 1996, substantial cultivation began of several kinds of GM crops, a harmful insect-resistant GM maize and an herbicide tolerant soybean. Since then, a total of 130 crop events in 22 plant species have been commercialized (Ramessar et al., 2008). The global area of GM crop-cultivation (mainly for maize soybean, canola and cotton) has been rapidly increasing (James, 2008). In 2008, there were 125 million hectares of GM crops in 25 countries: USA (62.5 million hectares), Argentina (21.0 million hectares), Brazil (15.8 million hectares), India (7.6 hectares), Canada (7.6 hectares), China (3.8 hectares), Paraguay (2.7 hectares), South Africa (1.8 million hectares), Uruguay (0.7 million hectares), Bolivia (0.6 million hectares), Philippines (0.4 million hectares), Australia (0.2 million hectares), Mexico (0.1 million hectares), Spain (0.1 million hectares), Chile (below 0.1 million hectares), Colombia (below 0.1 million hectares), Honduras (below million hectares), Burkina Faso (below 0.1 million hectares), Czech Republic (below 0.1 million hectares), Romania (below 0.1 million hectares), Portugal (below million hectares), Germany (below 0.1 million hectares), Poland (below 0.1 million hectares), Slovakia (below 0.1 million hectares), and Egypt (below 0.1 million hectares). There was a 9.4% increase in acreage from 2007 to 2008. The topic of this dissertation is stacked trait varieties, the GM crops in which two or more recombinant traits are introduced. The term “trait hectare” in Fig. 1.2 means the values obtained by multiplying the trait numbers by the cultivation area. In 2008, the trait hectares reached 166 million hectares, a 15.5% increase over 2007. Comparison of this increase to that of the original acreage (9.4%) indicates that adoption of the stacked trait varieties has increased. The USA led the way with 41% of its total 62.5 million hectares of biotech crops comprised of stacked trait varieties, including 75% of cotton and 78% of maize. The fastest growing component of stacked maize in the USA has been triple stacks, which confer resistance to two insect pests plus herbicide tolerance. A GM maize with eight genes,

called SmartStax, is expected to be released in the USA in 2010 with eight different genes coding for several insect-resistant and herbicide-tolerant traits (James, 2008).

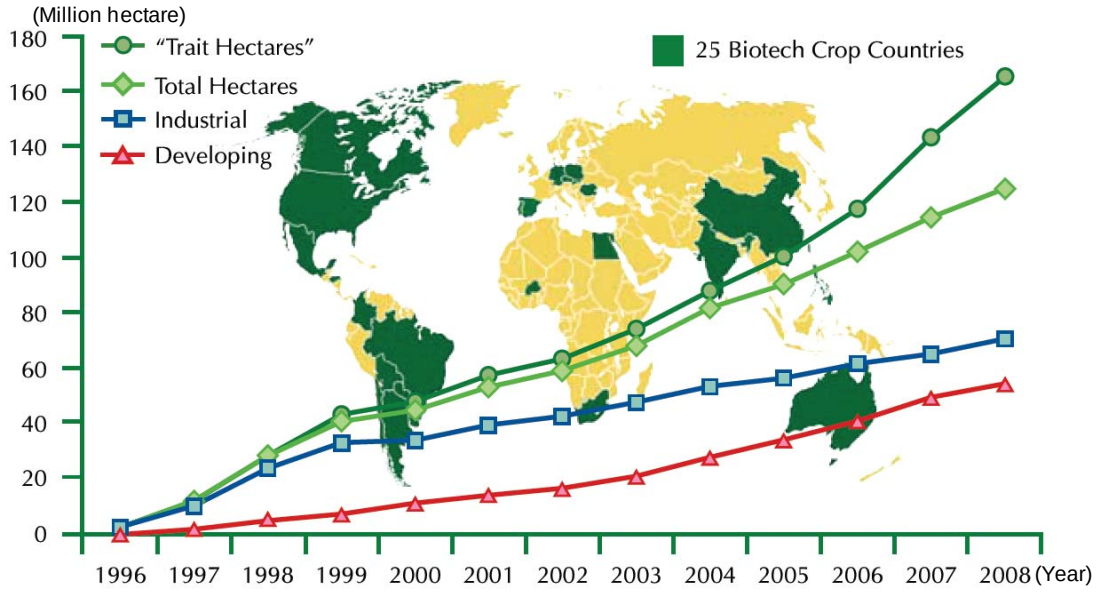

Fig. 1.2. Global area of GM crops. The graph indicates the global area (million hectares) of GM crop cultivation each year. The countries colored green on the global map are countries where GM crops are cultivated (source: James, 2008).

GM crops are currently classified using the word "generation" according to the objective of the trait being introduced. The first generation of GM crops refers to seeds that have been biotechnologically derived to increase production, but the crops themselves are not substantially different from their conventional counterparts. In other words, these crops are

similar to conventional crops either in appearance, taste, or nutritional value. The seeds have specific resistance mechanisms to combat herbicides and/or harmful insects. These crops are currently planted on millions of farmland hectares, as described above. The second generation of GM plants consists of crops with new traits of direct value to consumers. These crops have benefits to the processor, end user and consumer such as increased levels of nutrition or other phytochemicals. Some examples of these GM plants are rice with beta-carotene or higher iron and zinc levels, tomato with enhanced levels of carotenoids, flavonoids, and phenolics; maize with increased vitamin C levels, soybean with improved amino acid composition, or potato with enhanced calcium content (Magaña-Gómez and Calderón, 2008). The GM carnation which was modified in its pigment synthesis pathway is colored blue depending on the delphinidin from the modified pathway. This carnation is marketed under the trade name Moondust (Suntory Flowers Ltd, Chiyoda, Japan) and can be widely purchased as a cut flower. It was one of the second generations already commercialized.

Fig. 1.3. The genetically modified carnation, Moondust
(source: Suntory Flowers Ltd. website).

A third generation of GM plants is emerging from the research pipeline. Some of the genetic modifications in these plants are designed to confer a greater ability to resist abiotic stress such as drought, high temperatures, or saline soils. Other modified crops provide renewable energy sources. Furthermore, this third generation also includes plants which are used as biological production systems for manufacturing high-grade active pharmaceutical ingredients (Magaña-Gómez and Calderón, 2008).

The World Commission on the Environment and Development (held between 1984 and 1987) defined sustainable development as follows: "Sustainable development is development that meets the needs of the present without compromising the ability of future generations to meet their own needs." GM crops have contributed to sustainable development in several significant ways, as follows. First, GM crops can play an important role in contributing to food security and providing more affordable food through increasing supply (by increasing productivity per hectares) and, coincidently, decreasing cost of production (by a reduced need for inputs, less ploughing and fewer pesticide applications). These characteristics contribute to reducing the environmental footprint of agriculture such as CO_2 emissions, and this would help mitigate global climate change. In addition, increasing the efficiency of water usage will have a major impact on the conservation and availability of water globally. GM crop cultivation is also a land-saving technology, and can thus help reduce deforestation and protect biodiversity in forests. Furthermore, biotechnology can be used to cost-effectively optimize the productivity of biomass/hectare of energy crops. This can be achieved by developing crops tolerant to abiotic stresses and biotic stresses, and also by raising the ceiling of potential yield per hectare through modifying plant metabolism. Biotechnology can also be used to develop more effective enzymes for the downstream processing of biofuels (James, 2008).

There is currently a great deal of research taking place to develop second and third generation GM crops. These new generations of GM crops are expected to contribute

extremely to the sustainable development of agriculture. Concurrently, there is expected to be an increase in the number of commercialized GM events.

1.3 Regulations on genetic recombination and industrial use

After the first genetic recombination by Stanley N. Cohen and Herbert W. Boyer, scientists were confident the new technology offered considerable opportunities. However, they were only just learning how to manipulate DNA from various sources into combinations that were not known to exist naturally. Additionally, at that time the potential health and environmental risks were unclear (Berg, 2008). After about a year of moratorium on certain recombinant DNA experiments that were considered potentially hazardous, Paul Berg at the Massachusetts Institute of Technology held the Asilomar conference on recombinant DNA in 1975, in which scientists discussed the potential biohazards as well as the regulation of biotechnology. At this conference, it was proposed that the biological and physical containment of potentially biohazardous agents should be done according to the risks presented, and that the types of containment should be matched with the types of experiments performed (Berg et al., 1975). In 1976, the National Health Institute (NIH) released guidelines for genetic recombination which were based on the discussions by the experts at the Asilomar conference. The first guidelines strictly posed containment on scientists, but the guidelines have been modified and the regulations decreased due to the novel scientific findings about the safety of genetic recombination that have been presented in several international workshops.

In 1979, human growth hormone was produced from GM microorganisms, and the genetic recombination technique began to be applied for industrial use. In 1983, the Organization for Economic Co-operation and Development (OECD) created an ad hoc committee on the safety and regulation of biotechnology and the committee released the report “Recombinant DNA Safety Considerations” in 1986. In the report, it was stated that

the vast majority of industrial recombinant DNA large-scale applications would use organisms of intrinsically low risk, which could be used under only minimal containment consistent with good industrial large-scale practices (GILSP). In the report, permission was given for GM microorganisms similar to those that had been in industrial use for a long time to be used as same as those were handled. This report played a major role in expanding the possibilities for chemical or biomolecular production based on genetic recombination (Kato, 2005).

After deregulation of the guideline from the NIH about the open field-use of GMOs in 1982, there was considerable discussion about the safety of GMOs in open field use and the methodology of risk evaluation. Since release of the report "Recombinant DNA Safety Considerations" by the OECD in 1986, the basic concepts, i.e., "case by case", "step by step" and "familiarity", were established through some venues, such as a symposium held by the American Society for Microbiology titled "Engineered Organisms in the Environment: Scientific Issues" and reports from the National Academy of Sciences titled "Introduction of recombinant DNA –Engineered organisms into the environment: Key issues" and "Field testing genetically modified organisms: Framework for decisions'. "Case by case" is the concept that an individual review of a proposal regarding a kind of GMO should be performed under assessment criteria which are relevant to the particular proposal. "Step by step" is the concept that the trial and safety evaluation of individual GMOs should be performed in the following order: laboratory/greenhouse, small-scale trial, large scale trial, and open field trial. In 1992, the OECD's report "Safety considerations for biotechnology" described "Good Developmental Principles" (GDP) for field trial of plants and microorganisms with newly introduced traits (Kato, 2005). Finally, the OECD's report "Safety considerations for biotechnology: scale up for crop plants" in 1993 proposed the representative basic concept of familiarity, in which knowledge and experiences regarding the plant, the introduced trait, and the environment and their interaction may be applied to

facilitate risk/safety analysis and to manage possible risks in the context of scaling up GM plant-cultivation towards commercial release. The attitude for commercial release of GMOs was established by the report.

In the 1990s, discussions also began on the safety evaluation of GM foods. The OECD released a report titled "Safety evaluation of foods derived by modern biotechnology: Concepts and principles." In the report, “substantially equivalent,” the idea that existing organisms used as a source of food can be used as the basis for comparison when assessing the safety of human consumption of a food or food component that has been modified or is new, was proposed. This idea is still being applied to the safety assessment system in many countries (Kato, 2005).

In Japan, many regulations on GMOs that follow the international guidelines described above have been established since the 1980s. In 1991, the Ministry of Health, Labour and Welfare of Japan (MHLW) enacted guidelines for the safety evaluation of food and food additives produced by recombinant DNA techniques, and the Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF) enacted guidelines for the safety evaluation of feed produced by genetically modified organisms in 1996. A mandated system for enforcing the safety assessment of GM foods in Japan was introduced in 2001. Furthermore, Japan ratified the Cartagena Protocol on Biosafety in 2003 and the Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (the Cartagena protocol domestic law) was promulgated. The present Japanese safety assessment system for GMOs is summarized in Fig. 1.4. The companies developing new GMOs carry out the procedures for their commercialization under the safety assessment system, and only authorized GMOs have been allowed to be commercialized. The approved GM crops under the Japanese system are listed in Table 1.1. Furthermore, the food labeling system for GMOs was also implemented in 2001 under the Law Concerning Standardization and Proper Labeling of Agricultural and Forestry

Products (the Japanese agricultural standards law) in order to expand consumers' choices with regard to GM foods.

In the 2000s, individual countries or districts established regulations on the use of GMOs as food and feed materials. Because these regulations were determined by each country, they vary from country to country. For example, the thresholds for the GM food labeling system are 0.9%, 3% and 5% in the EU, Korea and Japan, respectively.

As seen from the information presented above, there has always been scientific discussion of genetic recombination and its industrialization, and many regulations have been derived from these discussions. There are numerous studies on the safe use of GMOs. These studies, as well as the experiences that have been accumulated using GMOs and the many established regulations for GMO control, such as safety assessment systems and food labeling systems, have enabled GMOs to be consumed, even as food materials. However, this situation has also led to public concern about the safety of GMOs and their environmental effects. Thus, the public administrations in charge of GMO regulations require scientific verification methods for appropriate implementation of regulations on GMOs. Along with the establishment of regulations, private food manufacturing or food distribution companies need GMO testing methods to comply with the regulations. There is thus a demand for GMO quantitation methods for the verification of an appropriate food labeling system in Japan. Additionally, for verification of the safety assessment system, methods to detect unapproved GMO contamination are needed. Thus, investigations of GMO testing methods have begun.

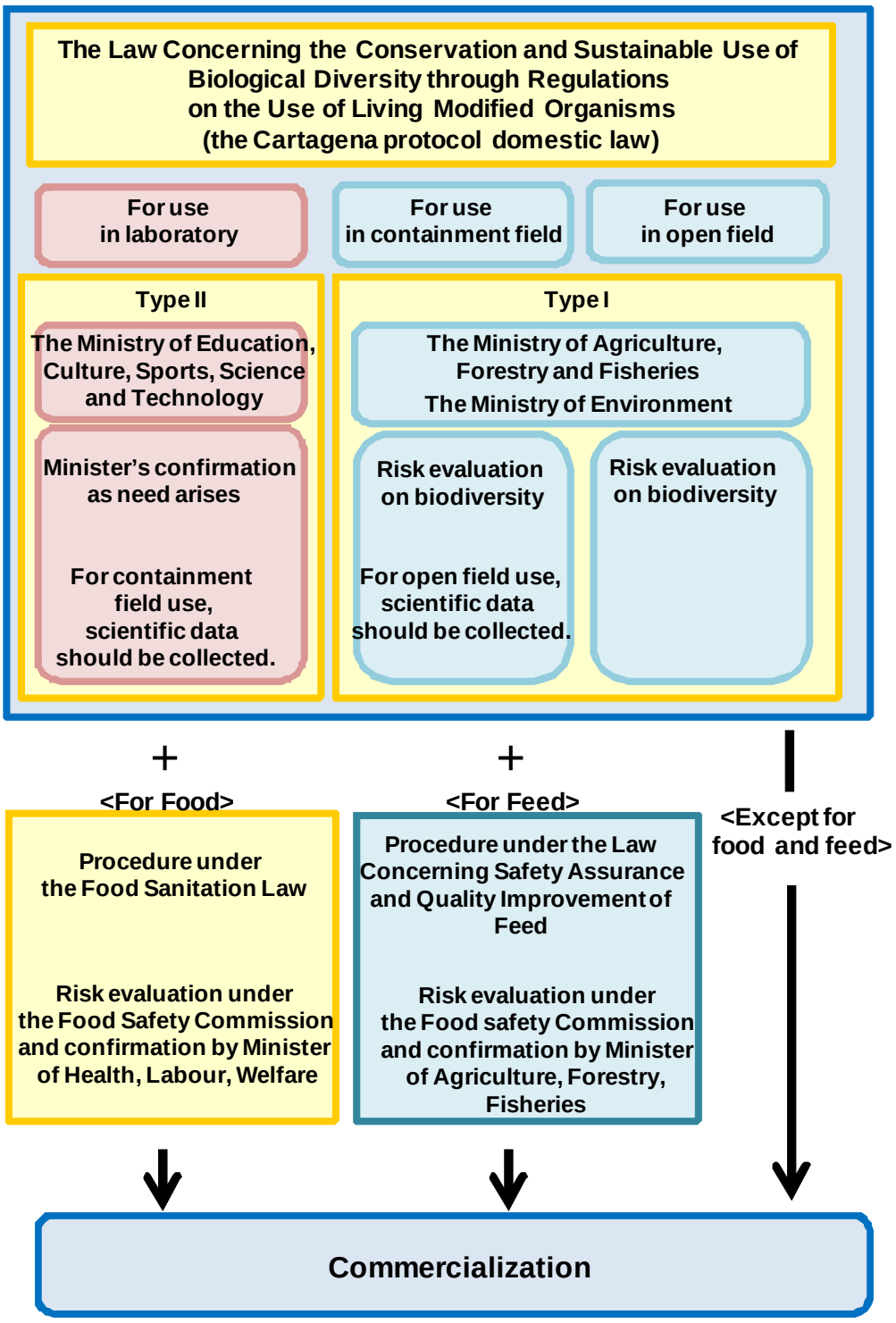

Fig.1.4. The safety assessment system for GMOs in Japan (source: Ministry of Agriculture, Forestry and Fisheries website).

Table 1.1. The list of approved GM crop events under the safety assessment systems in Japan (summarized from the websites of the Ministry of Agriculture, Forestry and Fisheries and the Ministry of Health, Labour and Welfare).

	No.	Event	Trait	Applicant	Risk evaluation on biodiversity (As of 8 July, 2009)				Food	Feed
					Open field cultivation	Use for food	Use for feed	Use for ornamental purpose	(As of 30 April, 2009)	(As of 8 June, 2009)
Alfalfa (*Medicago sativa*)	1	J101	Herbicide tolerant	Monsanto Japan, Ltd	2006	2006	2006	-	2005	2006
	2	J163	Herbicide tolerant	Monsanto Japan, Ltd	2006	2006	2006	-	2005	2006
	3	J101 x J163	Herbicide tolerant	Monsanto Japan, Ltd	2006	2006	2006	-	2005	2006
Carnation (*Dianthus caryophyllus*)	1	123.2.2	Pigment biosynthesis-modification	Suntory Holdings, Ltd	2004	-	-	2004	-	-
	2	11	Pigment biosynthesis-modification	Suntory Holdings, Ltd	2004	-	-	2004	-	-
	3	11363	Pigment biosynthesis-modification	Suntory Holdings, Ltd	2004	-	-	2004	-	-
	4	123.2.38	Pigment biosynthesis-modification	Suntory Holdings, Ltd	2004	-	-	2004	-	-
	5	123.8.8	Pigment biosynthesis-modification	Suntory Holdings, Ltd	2004	-	-	2004	-	-
	6	123.8.12	Pigment biosynthesis-modification	Suntory Holdings, Ltd	2009	-	-	2009	-	-
Cotton (*Gossypium hirsutum*)	1	BXN 10211	Herbicide tolerant	Stoneville Pedigreed Seed	-	-	-	-	2001	-
	2	BXN 10215	Herbicide tolerant	Stoneville Pedigreed Seed	-	-	-	-	2001	Approved
	3	BXN 10222	Herbicide tolerant	Stoneville Pedigreed Seed	-	-	-	-	2001	Approved
	4	1445	Herbicide tolerant	Monsanto Japan, Ltd	-	2004	2004	-	2001	2003
	5	531	Insect resistant	Monsanto Japan, Ltd	-	2004	2004	-	2001	2003
	6	15985	Insect resistant	Monsanto Japan, Ltd	-	2004	2004	-	2002	2003
	7	1445 x 531	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd	-	2004	2004	-	2003	2003
	8	15985 x 1445	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd	-	2005	2005	-	2003	2003
	9	757	Insect resistant	Monsanto Japan, Ltd	-	2005	2005	-	2001	2003
	10	LLcotton25	Herbicide tolerant	Bayer Crop Science, Ltd	-	2006	2006	-	2004	2006
	11	MON88913	Herbicide tolerant	Monsanto Japan, Ltd	-	2006	2006	-	2005	2006
	12	MON88913 x 15985	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd	-	2006	2006	-	2005	2006
	13	281	Herbicide tolerant Insect resistant	Dow chemical Japan, Ltd	-	-	-	-	2005	Approved
	14	3006	Herbicide tolerant Insect resistant	Dow chemical Japan, Ltd	-	-	-	-	2005	Approved
	15	281 x 3006	Herbicide tolerant Insect resistant	Dow chemical Japan, Ltd	-	2006	2006	-	2005	2006
	16	281 x 3006 x 1445	Herbicide tolerant Insect resistant	Dow chemical Japan, Ltd	-	2006	2006	-	2006	2006
	17	281 x 3006 x MON88913	Herbicide tolerant Insect resistant	Dow chemical Japan, Ltd	-	2006	2006	-	2006	2006
	18	LLcotton25 x 15985	Herbicide tolerant Insect resistant	Bayer Crop Science, Ltd	-	2007	2007	-	2006	2006
Maize (*Zea mays*)	1	MON810	Insect resistant	Monsanto Japan, Ltd	2004	2004	2004	-	2001	2003
	2	MON863	Insect resistant	Monsanto Japan, Ltd	2004	2004	2004	-	2002	2003
	3	MON810 x MON863	Insect resistant	Monsanto Japan, Ltd	2004	2004	2004	-	2004	2004
	4	NK603	Herbicide tolerant	Monsanto Japan, Ltd	2004	2004	2004	-	2001	2003
	5	T25	Herbicide tolerant	Bayer Crop Science, Ltd	2004	2004	2004	-	2001	2003
	6	NK603 x MON810	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd	2004	2004	2004	-	2003	2002
	7	MON863 x NK603 x MON810	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd	2004	2004	2004	-	2004	2004
	8	MON863 x NK603	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd	2004	2004	2004	-	2003	2003
	9	TC1507	Herbicide tolerant Insect resistant	Dupont K.K.	2005	2005	2005	-	2002	2003
	10	TC1507 x NK603	Herbicide tolerant Insect resistant	Dupont K.K.	2005	2005	2005	-	2004	2003
	11	GA21	Herbicide tolerant	Monsanto Japan, Ltd	2005	2005	2005	-	2001	2003
	12	T25 x MON810	Herbicide tolerant Insect resistant	Dupont K.K.	2005	2005	2005	-	2003	2001

Table 1.1. Continued.

	No.	Event	Trait	Applicant	Risk evaluation on biodiversity (As of 8 July, 2009)				Food	Feed
					Open field cultivation	Use for food	Use for feed	Use for ornamental purpose	(As of 30 April, 2009)	(As of 8 June, 2009)
Maize (*Zea mays*) continuied	13	GA21 x MON810	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd	2005	2005	2005	-	2003	2001
	14	T14	Herbicide tolerant	Bayer Crop Science, Ltd	-	2006	2006	-	2001	2005
	15	DAS-59122-7	Herbicide tolerant Insect resistant	Dupont K.K.	2006	2006	2006	-	2005	2006
	16	MON88017	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd	2006	2006	2006	-	2005	2006
	17	DLL25	Herbicide tolerant	Monsanto Japan, Ltd	2006	2006	2006	-	2001	2003
	18	MON88017 x MON810	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd	2006	2006	2006	-	2005	2006
	19	TC1507 x DAS-59122-7	Herbicide tolerant Insect resistant	Dupont K.K.	2006	2006	2006	-	2005	2006
	20	DAS-59122-7 x NK603	Herbicide tolerant Insect resistant	Dupont K.K.	2006	2006	2006	-	2005	2006
	21	DAS-59122-7 x TC1507 x NK603	Herbicide tolerant Insect resistant	Dupont K.K.	2006	2006	2006	-	2005	2006
	22	DBT418	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd	-	2007	2007	-	2001	2003
	23	Bt11	Herbicide tolerant Insect resistant	Syngenta seed K.K.	2007	2007	2007	-	2001	2003
	24	Bt11 sweet corn	Herbicide tolerant Insect resistant	Syngenta seed K.K.	-	-	-	-	2001	-
	25	Event176	Herbicide tolerant Insect resistant	Syngenta seed K.K.	2007	2007	2007	-	2003	2003
	26	LY038	High Lysine	Monsanto Japan, Ltd	2007	2007	2007	-	2007	2007
	27	MIR604	Insect resistant	Monsanto Japan, Ltd	2007	2007	2007	-	2007	2007
	28	Bt11 x GA21	Herbicide tolerant Insect resistant	Syngenta seed K.K.	2007	2007	2007	-	2007	2007
	29	MIR604 x GA21	Herbicide tolerant Insect resistant	Syngenta seed K.K.	2007	2007	2007	-	2007	2007
	30	LY038 x MON810	High Lysine Insect resistant	Monsanto Japan, Ltd	2007	2007	2007	-	2007	2007
	31	TC6275	Herbicide tolerant Insect resistant	Dow chemical Japan, Ltd	2008	2008	2008	-	2007	2007
	32	MON89034	Insect resistant	Monsanto Japan, Ltd	2008	2008	2008	-	2007	2007
	33	Bt11 x MIR604	Herbicide tolerant Insect resistant	Syngenta seed K.K.	2008	2008	2008	-	2007	2007
	34	Bt11 x MIR604 x GA21	Herbicide tolerant Insect resistant	Syngenta seed K.K.	2008	2008	2008	-	2007	2007
	35	MON89034 x MON88017	Insect resistant	Monsanto Japan, Ltd	2008	2008	2008	-	2008	2007
	36	MON89034 x NK603	Insect resistant	Monsanto Japan, Ltd	2008	2008	2008	-	2008	2007
	37	MON89034 x TC1507	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd Dow chemical Japan, Ltd	-	-	-	-	2008	Approved
	38	MON89034 x DAS-59122-7	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd Dow chemical Japan, Ltd	-	-	-	-	2008	Approved
	39	TC1507 x MON88017	Herbicide tolerant Insect resistant	Dow chemical Japan, Ltd Monsanto Japan, Ltd	-	-	-	-	2008	Approved
	40	DAS-59122-7 x MON88017	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd Dow chemical Japan, Ltd	-	-	-	-	2008	Approved
	41	MON89034 x TC1507 x MON88017	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd Dow chemical Japan, Ltd	-	-	-	-	2008	Approved
	42	MON89034 x TC1507 x DAS-59122-7	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd Dow chemical Japan, Ltd	-	-	-	-	2008	Approved
	43	MON89034 x DAS-59122-7 x MON88017	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd Dow chemical Japan, Ltd	-	-	-	-	2008	Approved
	44	1507 x DAS-59122-7 x MON88017	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd Dow chemical Japan, Ltd	-	-	-	-	2008	Approved
	45	MON89034 x DAS-59122-7 x MON88017	Herbicide tolerant Insect resistant	Monsanto Japan, Ltd Dow chemical Japan, Ltd	-	-	-	-	2008	Approved

Table 1.1. Continued.

	No.	Event	Trait	Applicant	Risk evaluation on biodiversity (As of 8 July, 2009)				Food	Feed
					Open field cultivation	Use for food	Use for feed	Use for ornamental purpose	(As of 30 April, 2009)	(As of 8 June, 2009)
Potato (*Solanum tuberosum*)	1	BT-6	Insect resistant	Monsanto Japan, Ltd	-	-	-	-	2001	-
	2	SPBT02-05	Insect resistant	Monsanto Japan, Ltd	-	-	-	-	2001	-
	3	RBMT21-129	Insect resistant Virus resistant	Monsanto Japan, Ltd	-	-	-	-	2001	-
	4	RBMT21-350	Insect resistant Virus resistant	Monsanto Japan, Ltd	-	-	-	-	2001	-
	5	RBMT22-82	Insect resistant Virus resistant	Monsanto Japan, Ltd	-	-	-	-	2001	-
	6	RBMT15-101	Insect resistant Virus resistant	Monsanto Japan, Ltd	-	-	-	-	2003	-
	7	SEMT15-15	Insect resistant Virus resistant	Monsanto Japan, Ltd	-	-	-	-	2003	-
	8	SEMT15-02	Insect resistant Virus resistant	Monsanto Japan, Ltd	-	-	-	-	2003	-
Rapeseed(canola) (*Brassica napus*)	1	RT73	Herbicide tolerant	Monsanto Japan, Ltd	2006	2006	2006	-	2001	2003
	2	RT200	Herbicide tolerant	Monsanto Japan, Ltd	2006	2006	2006	-	2001	2003
	3	MS8	Herbicide tolerant Male sterility	Bayer Crop Science, Ltd	2006	2006	2006	-	2001	2003
	4	RF3	Herbicide tolerant Recovering Male sterility	Bayer Crop Science, Ltd	2007	2007	2007	-	2001	2003
	5	Topas 19/2	Herbicide tolerant	Bayer Crop Science, Ltd	2007	2007	2007	-	2001	2003
	6	MS8 x RF3	Herbicide tolerant Male sterility Recovering Male sterility	Bayer Crop Science, Ltd	2007	2007	2007	-	2001	2003
	7	MS1 x RF1 (PGS1)	Herbicide tolerant Male sterility Recovering Male sterility	Bayer Crop Science, Ltd	-	2007	2007	-	2001	2003
	8	MS1 x RF2 (PGS2)	Herbicide tolerant Male sterility Recovering Male sterility	Bayer Crop Science, Ltd	-	2007	2007	-	2001	2003
	9	T45 (PHY23)	Herbicide tolerant	Bayer Crop Science, Ltd	2007	2007	2007	-	2001	2003
	10	Oxy-235	Herbicide tolerant	Bayer Crop Science, Ltd	2008	2008	2008	-	2001	2003
	11	HCN10	Herbicide tolerant	Bayer Crop Science, Ltd	-	-	-	-	2001	Approved
	12	HCN92	Herbicide tolerant	Bayer Crop Science, Ltd	-	-	-	-	2001	Approved
	13	PHY14	Herbicide tolerant	Bayer Crop Science, Ltd	-	-	-	-	2001	Approved
	14	PHY35	Herbicide tolerant	Bayer Crop Science, Ltd	-	-	-	-	2001	Approved
	15	PHY36	Herbicide tolerant	Bayer Crop Science, Ltd	-	-	-	-	2001	Approved
Rose (*Rosa hybrida*)	1	WKS82/130-4-1	Pigment biosynthesis-modification	Suntory Holdings, Ltd	2008	-	-	2008	-	-
	2	WKS82/130-9-1	Pigment biosynthesis-modification	Suntory Holdings, Ltd	2008	-	-	2008	-	-
Soybean (*Glycine max*)	1	40-3-2	Herbicide tolerant	Monsanto Japan, Ltd	2005	2005	2005	-	2001	2003
	2	A2704-12	Herbicide tolerant	Bayer Crop Science, Ltd	-	2006	2006	-	2002	2003
	3	A5547-127	Herbicide tolerant Male sterility	Bayer Crop Science, Ltd	-	2006	2006	-	2002	2003
	4	260-05	High oleic acid	Dupont K.K.	-	2007	2007	-	2001	2003
	5	MON89788	Herbicide tolerant	Monsanto Japan, Ltd	2008	2008	2008	-	2007	2007
	6	DP-356043-5	Herbicide tolerant	Dupont K.K.	2009	2009	2009	-	2009	2009
Sugar beet (*Beta vulgaris*)	1	H7-1	Herbicide tolerant	Monsanto Japan, Ltd	2007	2007	2007	-	2003	2005
	2	T120-7	Herbicide tolerant	Bayer Crop Science, Ltd	-	-	-	-	2001	Approved
	3	77	Herbicide tolerant	Monsanto Japan, Ltd	-	-	-	-	2003	Approved

The values as table elements indicate the year of approval. "Approved" is listed in place of the year when the approval year was not specified on the websites referenced.

1.4 Detection methods for genetically modified crops

1.4.1 Category of basal technologies

GMOs are almost all visually indistinguishable from non-GMOs, with the blue GM carnation as a notable exception. The presence of recombinant proteins or DNAs that have never existed in non-GMOs are the only signature of GMOs. Thus, the difference between GMOs and non-GMOs can be clarified by biomolecular interactions, such as antigen-antibody reactions and DNA-DNA hybridization. The protein-based detection methods are primarily based on the antigen-antibody reaction and are categorized as immunoassays, while the DNA-based detection methods are primarily based on a target-specific DNA amplification technique, polymerase chain reaction (PCR).

1.4.2 Protein-based detection methods

Most GM crop detection methods targeting recombinant proteins are immunoassays. They are used in clinical diagnostics targeting infectious diseases, pregnancy, tumor markers, and so on. In addition to these uses, immunoassays have been developed as precise analytical methods for food allergens, microbial pathogens, plant pathogens, agronomic traits of crops, soil or water pollutants and pesticides (Stave, 1999). There are many immunoassay formats, and the choice of format is dependent on the target molecule and application. For the detection of recombinant proteins in GM crops, enzyme-linked immunosorbent assay (ELISA) and immunochromatography are the most commonly used test formats (Grothaus et al., 2006).

The ELISA, which was developed by Eva Engvall and Peter Perlmann, is a sensitive and quantitative assay technique using an antibody or antigen fixed to a solid phase and enzyme-linked antibody; (Engvall and Perlmann, 1971; Voller et al., 1978). In general, an ELISA test is carried out on a plastic microtiter plate over several hours. Depending on the manner of detection, the ELISA is classified as either a competitive or sandwich ELISA. The

principles of the ELISA are shown in Fig. 1.5. In a competitive ELISA, unlabeled antibody is incubated in the presence of its antigen. The bound antibody/antigen mixtures are then added to an antigen-coated well (A1). The plate is washed to remove the unbound antigen/antibody complexes and antigens (A2). In this step, the more antigen in the sample, the less the antibody can bind to the antigen on the plate. The secondary antibody which is specific to the primary antibody and is linked with the enzyme is then added (A3). A substrate is added, and the remaining enzymes elicit a colorimetric or fluorescent signal (A4). In a sandwich ELISA, a known quantity of capture antibody is bound to the surface of an assay plate. The antigen-containing sample is applied to the plate (B1). The plate is washed to remove the unbound antigen (B2). The enzyme linked secondary antibody which is specific to the target antibody is added (B3). Finally, after washing the plate and adding enzyme substrate, the colorimetric or fluorescent signal is measured (B4).

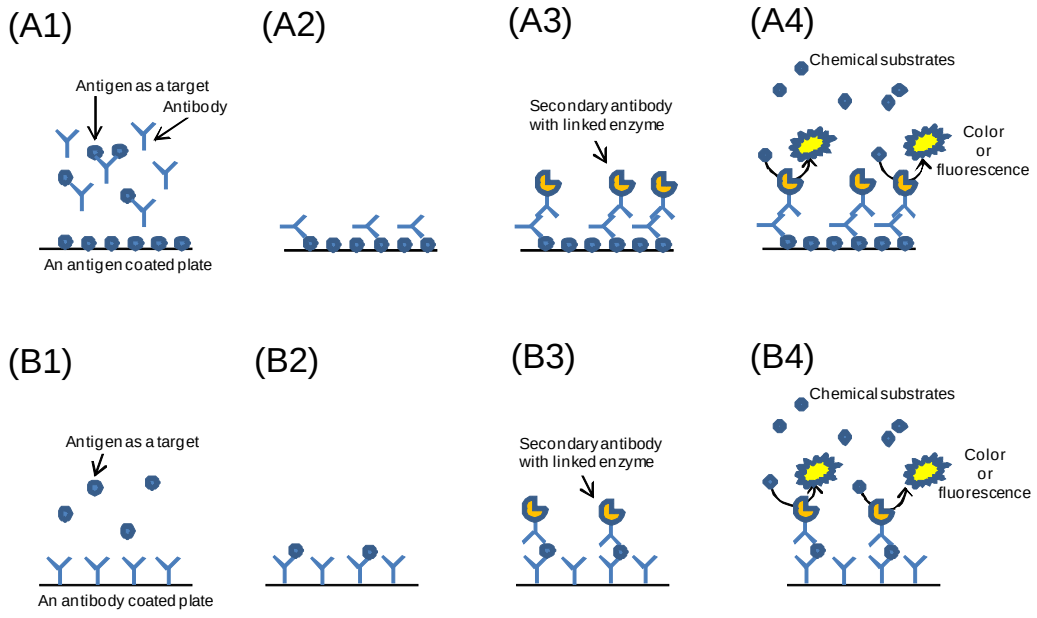

Fig. 1.5. The schematic representation of principles in the ELISA.
(A1-4), competitive ELISA; (B1-4), sandwich ELISA.

The immunochromatographic technique was first described in the late 1960's and is one of the immunoassay formats. The most advantageous characteristic is the rapid determination of the presence/absence of target molecules. Currently, sandwich type immunochromatography test kits, called a lateral-flow strip test, are primarily used. The principle of detection is shown in Fig. 1.6. The strip generally consists of a nitrocellulose membrane on a backing material with antigen-specific capturing antibody in a test line and an antibody in a control line. The detection antibody is conjugated to gold and dried onto a fiber pad. Optimized buffers necessary for the test performance are provided on the sample pad. The strip also contains a wicking pad made of fiber which provides the necessary wicking for the fluids to move through the membrane. When a positive sample is applied to the strip, the target antigen in the sample first binds to the gold-labeled antibody and flows through the membrane, forming a sandwich with the captured antibody present in the test line. This results in formation of a visible line, and the result is interpreted as positive. The excess gold-labeled antibody further moves and binds to the anti-detection antibody in the control line and the second line develops. This second line serves as an internal control. The analytical sample is negative for analyte if only the control line is present (Grothaus et al. 2006).

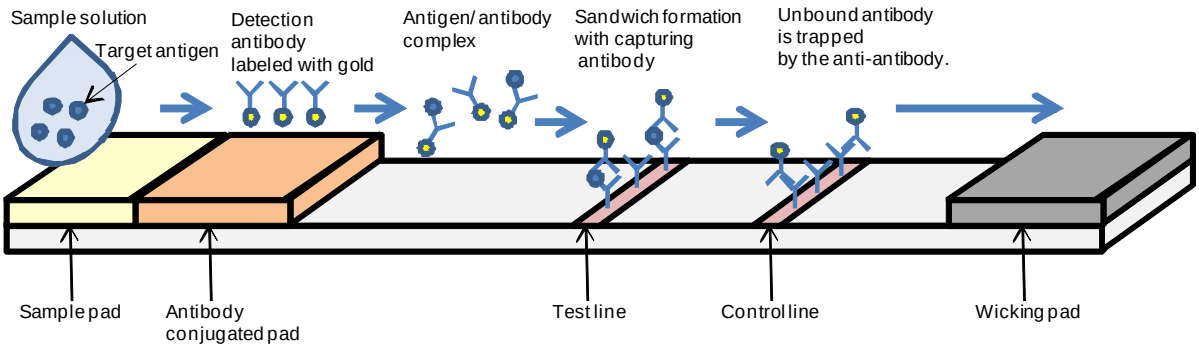

Fig. 1.6. Diagram of sandwich-type immunochromatography strip (referring to Grothaus, 2006).

ELISA and immunochromatography for GMO analyses have been developed and commercialized as test kits by several diagnostic test kit-developing companies, e.g., Strategic Diagnostics Inc. and EnviroLogix Inc. These kits target recombinant proteins in GM crops, such as phosphinothricin N-acetyltransferase from *Streptomyces viridochromogenes* (PAT), 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium tumefaciens CP4* (CP4-EPSPS) and insecticidal delta-endotoxins from *Bacillus thuringiensis* strains, CryIAb, CryIAc, CryIF, Cry2A, Cry3A, Cry3Bb, Cry9C and Cry34Ab1.

1.4.3 DNA-based detection methods

Most of the DNA-based GM crop detection methods exploit the polymerase chain reaction (PCR) technique. This technique, which was developed by Kary Mullis in 1983, allows amplification of a single or few copies of DNA, generating 10 million or more copies of a particular DNA sequence (Saiki et al., 1988). The DNA amplification relies on thermal cycling, consisting of repeated cycles of heating and cooling for DNA melting and enzymatic replication of the DNA, respectively. Almost all PCR applications employ a thermostable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus*. Primers, which are short DNA fragments containing sequences complementary to the target region, are key components enabling selective and repeated amplification. In addition to polymerase and primers, the DNA template includes a target sequence, four deoxyribonucleotides (deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxythymidine triphosphate and deoxycytidine triphosphate, generally described as dNTPs) and reaction buffer containing magnesium ion, all of which are indispensable for the PCR. As the PCR progresses, the generated DNA is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. Details of the amplification mechanisms are shown in Fig. 1.7. The PCR can extensively modify the performances of wide genetic manipulations because it

permits preparation of the necessary amount of DNAs with a specific nucleotide sequence.

The real-time PCR technique has been widely used for DNA-based GM crop quantitation. The technique is based on the instrumental real-time monitoring of fluorescent signals coupled with various kinds of chemistries for obtaining fluorescent signals dependent on DNA amplification. SYBR green and TaqMan chemistries are the most widely used chemistries (Gašparič et al., 2008). In SYBR green chemistry, SYBR green I dye, which specifically binds to double-stranded DNAs and emits fluorescence, is used for generating signals. Meanwhile, TaqMan chemistry is based on signal emission by cleavage of a dual-labeled fluorogenic probe (TaqMan probe) (Heid et al., 1996). The principle of TaqMan chemistry is shown in Fig. 1.8. The TaqMan probe does not emit fluorescence because the quencher dye reduces fluorescence by a quantum phenomenon, fluorescence resonance energy transfer (FRET) (Didenko, 2001). During extension of the nucleotide chain in PCR, TaqMan probes are cleaved by the 5'-3' exonuclease activity of Taq polymerase and the reporter dye emits a fluorescent signal. Real-time PCR with TaqMan chemistry (TaqMan PCR) shows higher specificity than conventional PCR and SYBR green chemistry-based real-time PCR because the chemistry demands precise annealing of the TaqMan probe with the template DNA. The disadvantage of TaqMan PCR is that additional synthesis of probes with a specific sequence for the target besides primers is required.

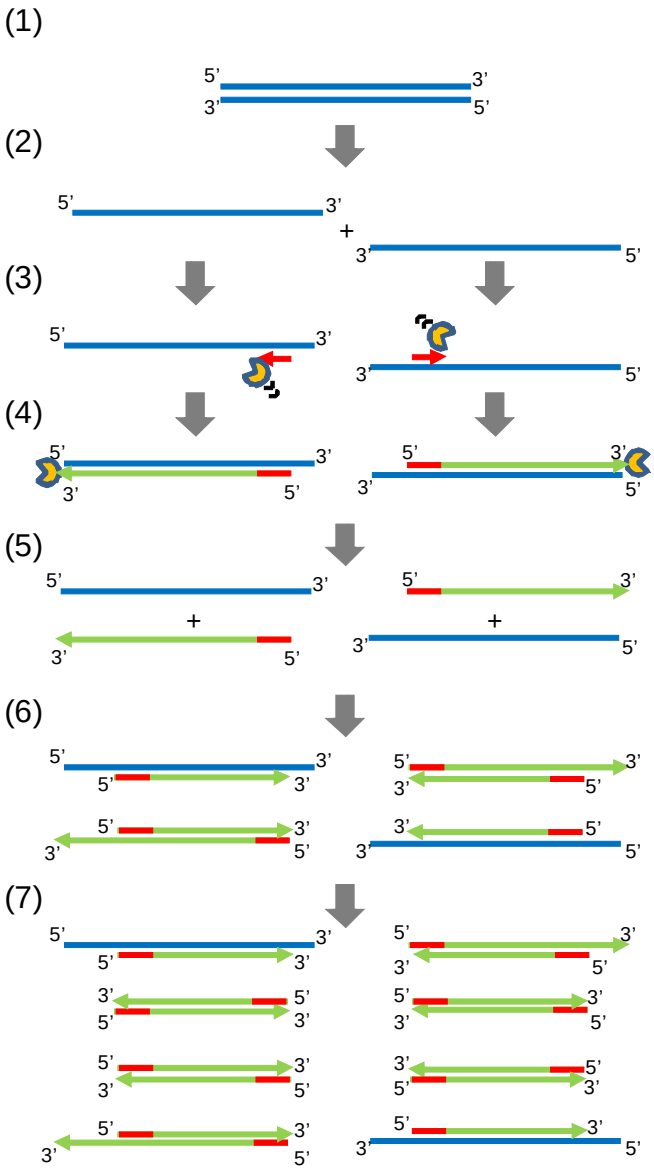

Fig. 1.7. Schematic representation of DNA amplification in polymerase chain reaction. Target DNA templates shown as blue bars form double strands in the reaction mixture (1). DNA templates are denatured at 94-96˚C (2). Oligonucleotide primers shown as red are annealed with their target region and are accompanied by DNA polymerases at a certain temperature below 65˚C (3). The primers are extended at about 72˚C (4). The products shown in the green arrow in the first cycle are denatured at 94-96˚C in the second cycle (5). The primers are annealed and extended in the second thermal cycle (6). In the third cycle, the second thermal cycle products are doubled (7). As the thermal cycles progress, target DNA are exponentially amplified.

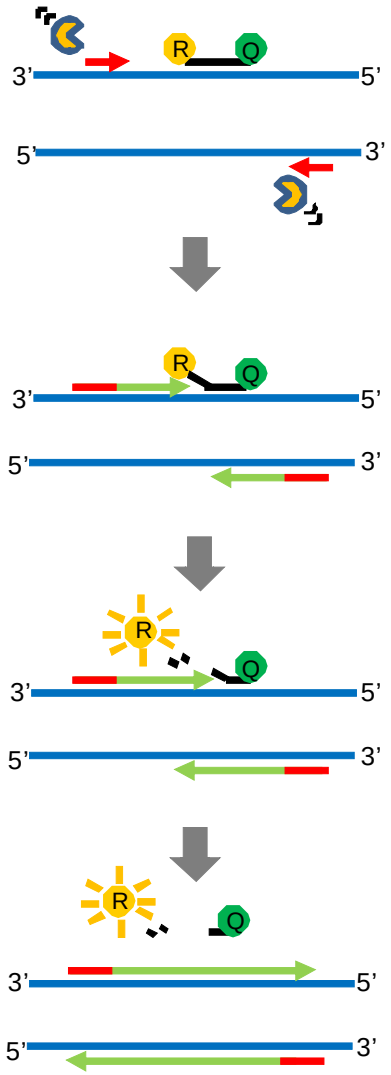

Fig. 1.8. Schematic representation of fluorescent signal generation in TaqMan PCR. Two primers and one TaqMan probe are annealed with a DNA template. DNA polymerase is indicated by a cracked circle. Reporter and quencher dyes are indicated as “R” and “Q”, respectively. During primer extension, the probe is hydrolyzed by the 5’-3’ exonuclease activity of DNA polymerase and fluorescence is generated from the reporter dye released from the probe.

One kind of GMO is defined by a unique transformation event. For example, MON809 maize and MON810 maize are considered two different GMOs, although both belong to the same species, *Zea mays*, and were transformed with the same plasmid (pV-ZMBK07) (Holst-Jensen, 2003). This is because the positions of the inserted DNAs in the maize genomes and a part of the recombinant nucleotide sequences are different between these two events.

The gene construct is composed of several recombinant DNA (r-DNA) segments, consisting usually of at least a promoter as a start signal, a gene of interest and a terminator functioning as a stop signal for regulation of gene expression. In addition, the construct may be flanked by DNA from the cloning vector. PCR-based GMO tests can be grouped into several categories (Fig. 1.9). Each category corresponds to the composition of the DNA fragment that is amplified in the PCR. Event-specific and construct-specific methods are candidates for GM events detection and identification. The only unique signature of a transformation event is the junction at the integration locus between the recipient plant genome and the inserted DNA. An event-specific method is defined as a method targeting the junction. A construct-specific method as an alternative targets the adjacent region of the gene construct, for example the region between the promoter and the trait gene. However, GM events with the same recombinant DNA constructs, such as MON809 and MON810, cannot be discriminated. For screening of GMOs, r-DNA segment detection is effective. The majority of GM plants have been transformed with constructs containing the Cauliflower Mosaic Virus (CaMV) 35S promoter (P35S) or the terminator region of nopalin synthase derived from *Rhizobium radiobacter* (TNOS). The most commonly used cloning vectors are pBR322 and its descendants (e.g., pUC19) containing a gene encoding neomycin phosphotransferase II (NPTII) for resistance to neomycin/kanamycin antibiotics. The NPTII gene as a selection marker is also useful for GM crop screening. In addition to these, trait genes commonly introduced into many GM events are also useful. However, these genes are

often slightly modified by, for example, truncation or altered codon usage. For the efficient screening of GM events, consensus regions in a trait gene need to be selected as the target region of the PCR. Even if the r-DNA segments are detected in the PCR detection method, it is suspicious that the source of the detected DNAs are CaMV and *Rhizobium radiobacter* naturally infected to plant bodies and adventitious existence of soil microorganisms. For reliable GM crop screening, donor organisms of r-DNA segments should not exist in the test materials. Therefore, detection methods specific for donor organisms of r-DNA segments are useful. Endogenous reference gene-detection is also important for GMO detection. In PCR testing, the positive control is needed for confirming well-performed DNA purification and DNA amplification. In most GMO quantitation strategies using real-time PCR, the copy number of the endogenous reference gene and r-DNA are compared and the GM crop content is then relatively determined.

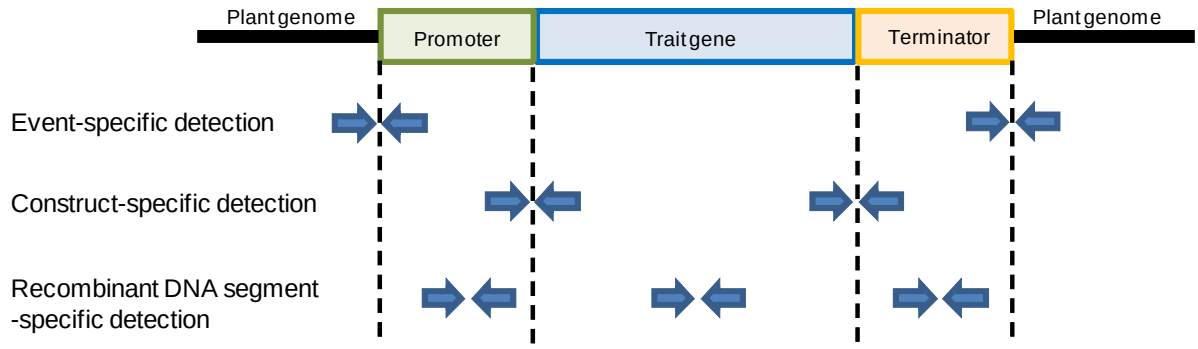

Fig. 1.9. Schematic representation of category of PCR detection methods.
Arrows indicates PCR primers.

In 1999, Matsuoka et al. reported a PCR-based detection method for a GM soy event, 40-3-2 (Roundup Ready soybean, RRS). Since then, many detection methods targeting

individual GM events or individual r-DNA segments have been developed with simplex PCR. These detection methods are listed in Table 1.3. A wide range of GM events in maize, soy, cotton, canola, and potato were the targets of these methods. In addition, a donor organism-specific detection method was developed for CaMV. Endogenous reference genes for maize, soy, canola, cotton, tomato, rice, wheat, eggplant and pepper have been developed. In addition to the methods shown in Table 1.3, many kinds of quantitative and event-specific real-time PCR methods were validated as official GMO detection methods under the European Commission [Community Reference Laboratory for GM Food and Feed (CRL-GMFF) website, http://gmo-crl.jrc.ec.europa.eu/].

As an increasing number of GMOs have been approved, there have been attempts to develop simultaneous detection methods for multiple targets to increase efficiency in GMO testing. The representative methods are shown in Table 1.4. Multiplex PCR coupled with the agarose gel electrophoresis technique has often been used. In 2005, a successful nonaplex (9-plex) PCR method was developed (Onishi et al., 2005). Though the number of GM events that can be targeted in such systems is increasing and the multiplex PCR strategy is efficient, multiplex PCR methods for more than nine targets have not been developed. This suggests that it is difficult to establish highly multiplexed methods. Besides the agarose gel-based multiplex PCR methods, the multiplex PCR and capillary gel electrophoresis method have been developed. In the strategy, a higher resolution of signal separation was achieved using capillary type electrophoresis and fluorescent dye-labeled primers. Further multiplex detection techniques such as PCR tandem with DNA microarray hybridization (including membrane hybridization and peptide nucleic acid (PNA) microarray-hybridization methods following multiplex PCR) have been investigated. A multiplex detection system for 28 targets was developed (Leimanis et al., 2006). For the efficient quantification of GM crops, many multiplex real-time PCR methods were also developed.

Table 1.3. Representative GM crop detection methods based on simplex PCR

Qualitative

Assay	Detection type	Targets	References
PCR and agarose gel analysis	Event-specific	CBH351 maize	Windels et al., 2003
		T25 maize	Collonier et al., 2005
		MON863 maize	Yang et al., 2005a
		MON531 cotton	Yang et al., 2005b
		MON1445 cotton	Yang et al., 2005b
		TC1507 maize	La paz et al., 2006
		T45 canola	Yang et al., 2006
		Bt11 maize	Yang et al., 2007a
		Event176 maize	Yang et al., 2007a
		MON810 maize	Yang et al., 2007a
		NK603 maize	Yang et al., 2007a
		CBH351 maize	Yang et al., 2007a
		RT73 canola	Pan et al., 2007
		GT73 canola	Yang et al.,2007b
		MS1×RF1 canola	Wu et al., 2007
		MS1×RF2 canola	Wu et al., 2007
		Oxy235 canola	Yang et al., 2008
		GA21 maize	Oguchi et al., 2008
	Construct-specific	Roundup Ready soybean	Matsuoka et al., 1999
		CBH351 maize	Matsuoka et al., 2001
		Bt11 maize	Kuribara et al., 2002
		Event176 maize	Kuribara et al., 2002
		GA21 maize	Kuribara et al., 2002
		MON810 maize	Kuribara et al., 2002
		T25 maize	Kuribara et al., 2002
		SEMT15-15 potato	Watanabe et al., 2004
		SEMT15-02 potato	Watanabe et al., 2004
		RBMT15-101 potato	Watanabe et al., 2004
	r-DNA segment-specific	GUS	Goda et al., 2001
		P35S	Kuribara et al., 2002
		TNOS	Kuribara et al., 2002
		35S terminator derived from CaMV	Matsuoka et al., 2002
		PAT	Matsuoka et al., 2002
		BAR	Matsuoka et al., 2002
		CryIA	Matsuoka et al., 2002
		m-EPSPS (modified maize enol pyruvate)	Matsuoka et al., 2002
		CP4-EPSPS	Matsuoka et al., 2002
		GOX	Matsuoka et al., 2002
		NPTII	Matsuoka et al., 2002
		Rice actin promoter	Matsuoka et al., 2002
		Cry9C	Windels et al., 2003
	Endogenous reference gene-specific	Acetyl-CoA carboxylase (canola)	Hernandez et al., 2001
		Starch synthase IIb (maize)	Kuribara et al., 2002
		Lectin1 (soy)	Kuribara et al., 2002
		Uridine diphosphate-glucose pyrophosphorylase (UGPase) (potato)	Watanabe et al., 2004
		Sucrose phospahte synthase (rice)	Ding et al., 2004
		Waxy-D1 (wheat)	Iida et al., 2005
		High mobility group protein (canola)	Weng et al., 2005
		Stearoyl-acyl carrier protein desaturase (Sad1) (cotton)	Yang et al., 2005c
		Putative protein Lat52 (tomato)	Yang et al., 2005d
	Donor organism-specific	CaMV	Chaouachi et al., 2008b
Real-time PCR (SYBR)	Event-specific	CBH351 maize	Windels et al., 2003
	r-DNA segment-specific	Cry9C	Windels et al., 2003
Real-time PCR (TaqMan)	r-DNA segment-specific	CryIAb	Vaïtilingom et al., 1999
	Event-specific	Bt11 maize	Ronning et al., 2003

Table 1.3. Continued.

Quantitative

Assay	Detection type	Targets	References
Competetive PCR	Construct-specific	RR soybean	Shimizu et al., 2008
	r-DNA segment-specific	P35S	Mavropoulou et al., 2005
	Endogenous reference gene-specific	Lectin1 (soy)	Mavropoulou et al., 2005
		Lectin1 (soy)	Shimizu et al., 2008
Real-time PCR (TaqMan)	Event-specific	Bt11 maize	Ronning et al., 2003
		MON810 maize	Huang et al., 2004
		NK603 maize	Huang et al., 2004
		T25 maize	Weighardt et al., 2004
		MON863 maize	Yang et al., 2005a
		T25 maize	Collonier et al., 2005
		TC1507 maize	La paz et al., 2006
		T45 rapeseed	Yang et al., 2006
		Bt11 maize	Yang et al., 2007a
		Event176 maize	Yang et al., 2007a
		MON810 maize	Yang et al., 2007a
		NK603 maize	Yang et al., 2007a
		CBH351 maize	Yang et al., 2007a
		GT73 canola	Yang et al.,2007b
		MS1 ×RF1 canola	Wu et al., 2007
		MS1 ×RF2 canola	Wu et al., 2007
		GA21 maize	Oguchi et al., 2008
		Oxy235 canola	Yang et al., 2008
	Construct-specific	Bt11 maize	Kuribara et al., 2002
		Event176 maize	Kuribara et al., 2002
		GA21 maize	Kuribara et al., 2002
		MON810 maize	Kuribara et al., 2002
		T25 maize	Kuribara et al., 2002
	r-DNA segment-specific	CryIAb	Vaïtilingom et al., 1999
		P35S	Kuribara et al., 2002
		TNOS	Kuribara et al., 2002
	Endogenous reference gene-specific	Zein (maize)	Vaïtilingom et al., 1999
		Acetyl-CoA carboxylase (canola)	Hernandez et al., 2001
		Starch synthase IIb (maize)	Kuribara et al., 2002
		Lectin1 (soy)	Kuribara et al., 2002
		Alcol dehydrogenase1 (maize)	Hernandez et al., 2004
		High-mobility group protein a (maize)	Hernandez et al., 2004
		Invertase1 (maize)	Hernandez et al., 2004
		Zein (maize)	Hernandez et al., 2004
		Sucrose phospahte synthase (rice)	Ding et al., 2004
		Stearoyl-acyl carrier protein desaturase (Sad1) (cotton)	Yang et al., 2005b
		Putative protein Lat52 (tomato)	Yang et al., 2005d
		Waxy-D1 (wheat)	Iida et al., 2005
		High mobility group protein (canola)	Weng et al., 2005
		Beta-fluctosidase (potato)	Chaouachi et al., 2008a
		Beta-fluctosidase (tomato)	Chaouachi et al., 2008a
		Beta-fluctosidase (eggplant)	Chaouachi et al., 2008a
		Beta-fluctosidase (pepper)	Chaouachi et al., 2008a
	Donor organism-specific	CaMV	Canker et al., 2005
		CaMV	Chaouachi et al., 2008b

Table 1.4. Representative multiplex detection methods for GM crops.

Qualitative

Assay	The number of multiplexed targets	Targets	References
PCR and agarose gel analysis	2	40-3-2 soy and Le1 (soy)	Hurst et al., 1999
	2	CryIAb and invertase1 (maize)	Hurst et al., 1999
	5	Bt11, Event176, MON810, T25/T14 maize and SSIIb (maize)	Matsuoka et al., 2000
	6	Bt11, Event176, GA21, MON810, T25 and SSIIb (maize)	Matsuoka et al., 2001
	2	PAT and CryIAb	Permingeat et al., 2002
	4	CryIAb, PAT, BAR, invertase (maize)	James et al., 2003
	5	Beta-actin, P35S, CP4-EPSPS, TNOS and lectin1 (soy)	James et al., 2003
	6	GOX, oxy, barnase, PAT, cruciferin (canola), chroloplast (plant)	James et al., 2003
	7	Bt11, GA21, MON810 maize, CryIAb (for Event176 maize), 40-3-2 soy, zein (maize), Le1 (soy)	Germini et al., 2004
	9	Bt11, Event176, GA21, MON810, MON863, NK603, T25, TC1507 maize and SSIIb (maize)	Onishi et al., 2005
	2	55-1 papaya and papain (papaya)	Yamaguchi et al., 2006
	9	Bt11, Event176, GA21, MON810, MON863, NK603, T25, TC1507 maize and zein (maize)	Shrestha et al., 2008
PCR and capilally gel electrophoresis	5	Bt11,GA21, MON810, NK603 maize and alcohol dehydrogenase1 (maize)	Nadal et al., 2006
	9	Bt11, Event176, GA21, MON810, MON863, NK603, T25, TC1507 maize and high mobility group protein (maize)	Heide et al., 2008
PCR coupled with array hybridization	9 (9 target on membrane)	P35S , TNOS, NPTII, PFMV, CryIAb, CP4-EPSPS, CryIIIA, BAR and 18SrRNA (plant)	Su et al., 2003
	4 (17 target on microarray)	40-3-2 soy, Le1 (soy) and 18SrRNA (plant)	Xu et al., 2005
	5 (17 target on microarray)	TNOS, BAR, CryIAb, invertase (maize) and 18SrRNA (plant)	Xu et al., 2005
	7 (7 targets on PNA chip)	Bt11, Event176, GA21, MON810 maize, 40-3-2 soy, SSIIb (maize), and Le1 (soy)	Germini et al., 2005
	21 (21 targets on microarray)	P35S, 35S terminator, NOS promoter, TNOS, PFMV, NPTII, BAR, PAT, CaMV cort protein, Le1 (soy), CP4-EPSPS, Rbc1 (plant), fbp, GOX, barnase, barstar, CryIAb, CryIAc, Cry9C, zein (maize), 18SrRNA (plant)	Xu et al., 2006
	28 (28 targets on microarray)	Bt11, E176, GA21, MON810, T25, CBH351 maize, 40-3-2 soy, Oxy235, T45, Topas canola, P35S, TNOS, NPTII, PAT, endogenous reference genes for maize, soy, canola, sugarbeet, tomato, CaMV	Leimanis et al. 2006
	12 (12 targets on microarray)	Bt11, Bt176, GA21, MON810, MON863, T25 maize,40-3-2 soy, P35S, TNOS, PFMV, NPTII, BAR, 18SrRNA (plant), CaMV	Xu et al., 2007
	10 (30 targets on maicroarray)	P35S, PFMV, TNOS, T35S, CP4-EPSPS, BAR, Oxy, NPTII, PAT, crusiferin (canola)	Shumidt et al., 2008
PCR coupled with ligation detection reaction and hybridization	2 (4 targets on microarray)	CryIAb and zein (maize)	Bordoni et al., 2004
	7 (7 targets on microarray)	Bt11, Event176, GA21, MON810 maize, 40-3-2 soy, zein (maize) and Le1 (soy)	Peano et al., 2005
	13 (14 targets on microarray)	Bt11, E176, GA21, MON810 maize, 40-3-2 soy, zein (maize) and Le1 (soy)	Bordoni et al., 2005
Real-time PCR (SYBR green)	2	GA21 and CryIAb for Event176 maize	Hernandez et al., 2003
	2	40-3-2 soy and Le1 (soy)	Hernandez et al., 2003
Ral-time PCR (TaqMan)	3	GA21 maize, P35S and SSIIb (maize)	Akiyama et al., 2005

Table 1.4. Continued.

Quantitative

Assay	The number of multiplexed targets	Targets	References
Real-time PCR (TaqMan)	2	P35S and zein (maize)	Hohne et al., 2002
	2	40-3-2 soy and Le1 (soy)	Foti et al., 2006
	2	P35S and TNOS	Waiblinger et al., 2006
Multiplex quantitative DNA array based PCR (MQDA-PCR)	8 (8 targets on microarray)	Bt11, Event176, MON810 maize, P35S, TNOS, internal positive control and an endogenous reference gene for maize	Rudi et al., 2003

For accurate DNA analysis, genomic DNA extraction and purification from the target plant body is indispensable. This is because nucleases, polyphenols, and certain kinds of protein contaminated in DNA samples may affect DNA quantification using ultraviolet spectroscopy and inhibit in vitro enzymatic reactions such as PCR. Due to the progress that has been made in molecular biology, a nucleotide purification technique has been developed. The basic strategy is comprised of detergent-mediated lysis, proteinase treatment, extractions with organic solvents, and ethanol precipitation. Polysaccharides and tannins in the plant body are difficult to separate from DNA. In 1980, a nucleotide purification technique from a plant body using cetyl trimethyl ammonium bromide (CTAB) was developed (Murray and Thompson, 1980). In 1989, a silica-based method was developed. The method is based on the lysing and nuclease-inactivating properties of the chaotropic agent, guanidinium thiocyanate, together with the nucleic acid–binding properties of silica particles in the presence of the agent (Boom et al., 1990). The method is rapid, simple, and reduces the probability of contamination between samples.

1.4.4 Comparison between protein and DNA-based methods

Protein-based assay methods have the advantage of easy pretreatment of analytical samples. However, it is difficult to obtain accurate results from heat-treated and processed

samples because antibodies do not bind to denatured or unfolded target recombinant proteins. Furthermore, the immunoassay is not adapted to certain GM events. For example, GA21 maize is one of the herbicide-tolerant GM events that have been broadly cultivated. The recombinant protein specifically expressed in GA21 is modified 5-enolpyruvylshikimate-3-phosphate synthase (mEPSPS), which was created by point mutation of original maize proteins, and thus preparation of an antibody specific for the recombinant protein has not been achieved. Additionally, it is reported that false-negative results tend to occur when the target proteins in test materials are present in too high of a concentration (Butch, 2000).

On the other hand, DNA-based detection methods such as PCR and real-time PCR enable highly specific detection but the DNA preparation is time-consuming. DNA-based methods are basically applicable for all GM events. Thus, the DNA-based methods are often advantageous in terms of reliability and applicability.

1.5 Application of detection methods for genetically modified crops

Many countries and regions of the world have established their own management system and regulations for GM crops. Thus, standard detection methods suitable for the regulations are often established by the respective public organizations. In Japan, standard GM crop-detection methods for the purpose of monitoring the food labeling system were developed and published as the Japanese Agricultural Standard (JAS) analytical test handbook from the MAFF and "Testing for Foods Produced by Recombinant DNA Techniques" from the MHLW. The testing methods for food, including unapproved GM crops, are also described in "Testing for Foods Produced by Recombinant DNA Techniques." In Europe, the official detection methods are published on the website of the Community Reference Laboratory on GM Food and Feed. Standard GM crop-detection methods are used for administrative monitoring. Because of the reliability of DNA-based methods, most of the

standard methods for regulation are DNA-based.

GM crop detection methods are also used for quality control of food and feed materials and seeds which are commercially distributed. In this case, protein-based detection methods have also proved useful because the test results can be quickly obtained.

The testing results obtained from GM crop-detection methods used for administrative monitoring under regulations directly affect commercial distribution. In particular, bilateral disputes may arise concerning GM crops that are traded internationally. Therefore, the analytical performance of detection methods and the quality assurance of these methods are important. Guidelines for method validation have been proposed by the International Standard Organization (ISO) and International Union of Pure and Applied Chemistry (IUPAC). To date, many kinds of GMO detection methods have been validated and are used as standard methods. Validation of these methods was often performed by many testing laboratories as collaborative studies.

1.6 Objectives and outline of dissertation

Due to the well organized regulations on GMOs in many countries and consumers' growing concerns about GM foods, there is demand for development of GM crop detection methods. Bioanalytical techniques such as ELISA, immunochromatography, PCR and real-time PCR have been applied to detect GM crops. Many simplex detection methods targeting GM crops have been established, and their analytical performances evaluated. Some of these methods have been adopted as standard detection methods and have mainly been used in administrative monitoring. PCR-based detection methods in particular have been the mainstream of such methods because of their specificity and versatility, as well as their applicability to all GM events. In addition, they can be used for various kinds of analytical samples including plant species, parts of the plant body tested, and samples that are processed or non-processed. The number of GM crops approved under safety assessment systems has

been increasing, and is predicted to continue to rapidly increase with the arrival of new second and third generation GM crop events. Given this, there has been a strong demand for efficient testing methods for GM crops so that simultaneous detection techniques targeting for GM crop events and/or r-DNA segments, such as multiplex PCR and multiplex PCR coupled with microarray hybridization, have been investigated. The techniques that have already been developed, however, do not have sufficient analytical performance and practical utility. These methods are difficult to update, although multiple target-detection systems should be updated more easily. In this dissertation, the author investigated novel strategies for the simultaneous detection of GM crops with the aim of developing comprehensive GM crop detection methods with high practical utility.

The management of unapproved GM crops is one of the most important purposes of GM crop detection methods. However, no perfect detection system for all unapproved GM crops has yet been developed, because there are no biological or chemical characteristics specific to such crops. The author attempted to utilize comprehensive detection methods as a means to manage unapproved GM crops.

There are two main topics of this study. The first is the development of a screening method for the detection of GM crops, and the second is the development of a universal platform that can be used for comprehensive GM crop detection. The outline of the dissertation is summarized in Fig. 1.10.

In Chapter 2, the author investigated a screening method for GM crop detection targeting some r-DNA segments. Some r-DNA segments have been commonly introduced into some GM events, e.g., P35S, TNOS and NPTII. Simultaneous detection of such r-DNA segments using multiplex reactions enables screening and profiling of the GM crops. In addition, simultaneous detection is expected to detect endogenous reference genes as the experimental control. For these purposes, an attempt was made to use the ligase chain reaction (LCR), which has higher specificity for target nucleotide sequences than PCR. The

LCR mechanism enables little or no interaction between individual reactions in the multiplex system. Therefore, high updatability of the total system is expected.

In Chapter 3, a universal platform for comprehensive GM crop detection was developed. To obtain exhaustive information on GM crops, individual GM events, r-DNA segments, endogenous reference genes, and donor organisms were designed as the targets of the system. The simultaneous implementation of simplex real-time PCR reactions was adopted to achieve high updatability. Furthermore, a comprehensive detection platform was utilized for the systematic assumption of unapproved GM crop detection.

In Chapter 4, the study is summarized and the conclusion of this dissertation presented.

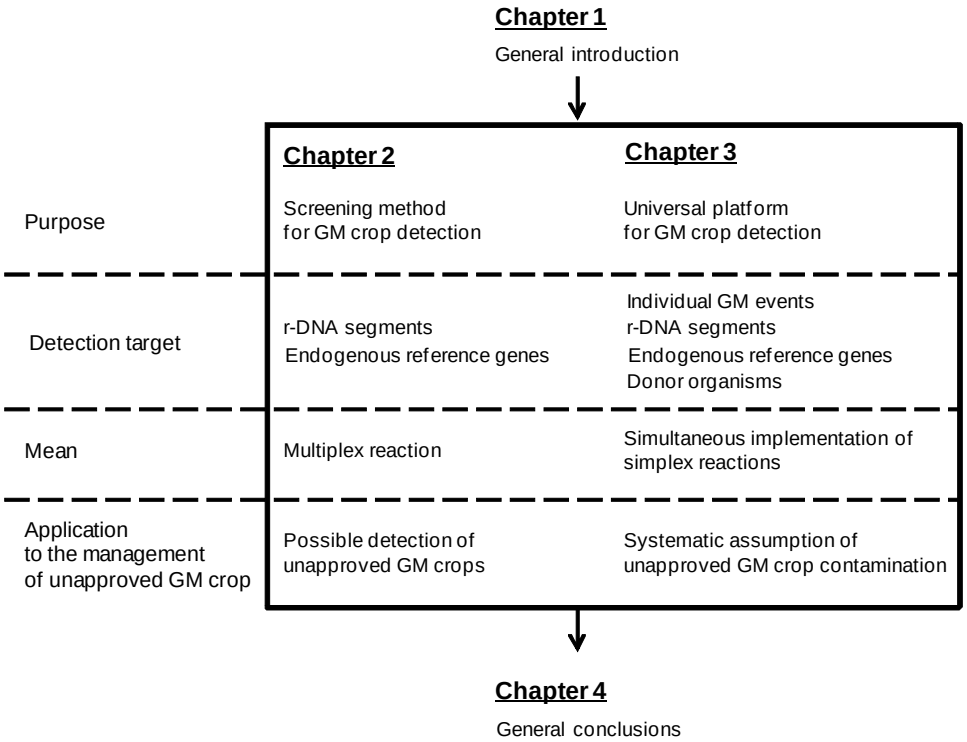

Fig. 1.10. Outline of dissertation.

Chapter 2

Screening method for the detection of genetically modified crops using multiplex ligase chain reaction coupled with multiplex polymerase chain reaction

2.1 Introduction

The acreage of commercial cultivation of genetically modified (GM) crops has been continuously increasing, and the number of countries engaging in GM cultivation grew to 25 in 2008 (James, 2008). Despite some opposition, GM crops are generally considered important sources of food and feed. Additionally, the production of biofuels such as ethanol and diesel oil is ongoing, and GM crops are expected to be a source of such fuels (Torney et al., 2007). Under these circumstances, novel types of GM crops are being developed all over the world, and the number of GM events obtaining regulatory approval in certain countries is increasing. Until now, GM event-specific detection methods have been developed and widely used for regulatory purposes. For example, many event-specific quantitative methods have been authorized and published under European Commission (Community Reference Laboratory for GM Food & Feed website, http://gmo-crl.jrc.ec.europa.eu/). Such circumstances have made the monitoring and control of GM crops more difficult, because the enforcement of event-specific detection for all approved events would not be realistic. Thus, testing methods which are capable of providing a great deal of information on GM crops and applicable for many types of GM crops are in demand.

Multiplex polymerase chain reaction (PCR) methods (Matsuoka et al., 2000; Matsuoka et al., 2001; James et al., 2003; Onishi et al., 2005) have been developed and reported as simultaneous detection tools for GM crops, as have DNA chip analyses (Rudi et al., 2003; Bordoni et al., 2004; Bordoni et al., 2005; Germini et al., 2005; Peano et al., 2005; Xu et al., 2005; Xu et al., 2006; Leimanis et al., 2006; Schmidt et al., 2008) and membrane-hybridization methods (Su et al., 2003). Although gel-based multiplex PCR assay is one of the most efficient techniques, such a method is difficult to develop for practical use because false-positive amplifications tend to occur more often than in simplex reactions (Elnifro et al., 2000; Markoulatos et al., 2002; Schmidt et al., 2008). Ligase chain reaction (LCR) has been studied as a specific DNA detection technique and applied for clinical

inspections such as the detection of pathogenic microorganisms and the discrimination of point mutations in human genomic DNA (Barany F., 1991a; Barany F., 1991b; Wiedmann et al., 1992; Laffler et al., 1993; Dille et al., 1993; Wiedmann et al., 1994; Lee et al., 1996; Freise et al., 2001; Schweitzer et al., 2001). The LCR amplification mechanism is shown in Fig. 2.1. LCR is a DNA amplification method that depends on the activity of thermostable ligase, and the amplification mechanism requires four LCR probes designed in adjacent and complementary positions in one target DNA region. In the thermal cycling of LCR, the four LCR probes anneal at the target sequence on the denatured DNA strands and two adjacent probes are linked by a thermostable DNA ligase (Lee, 1996). The ligated probes serve as targets for the subsequent cycles, leading to exponential amplification. LCR amplification has been reported to demand complete complementarity at the junction of the adjacent probes (Barany, 1991). Therefore, detection methods using the LCR technique are expected to have higher specificity for target nucleotide sequences than PCR amplification. In addition, for the simultaneous detection of tandemly arranged recombinant DNA (r-DNA) regions such as the multiplex detection of a promoter, a trait gene and a terminator in one cassette, multiplex LCR may provide further advantages over multiplex PCR. This is because multiplex LCR produces the amplified products with the specific lengths even for the adjacent targets, while multiplex PCR may produce unexpected amplified products containing multiple target regions.

In this chapter, multiplex LCR is applied for the simultaneous detection of target DNA regions and multiplex PCR is adopted as an efficient pre-amplification technique in order to obtain higher sensitivity in this novel system. The newly designed multiplex PCR-multiplex LCR (MPCR-MLCR) systems were intended for the simultaneous detection of r-DNA segments commonly introduced into some GM crop events and that of endogenous DNA segments as a positive control test. The systems would be useful for the screening detection of GM crops. An overview of the MPCR-MLCR technique is shown in Fig. 2.2.

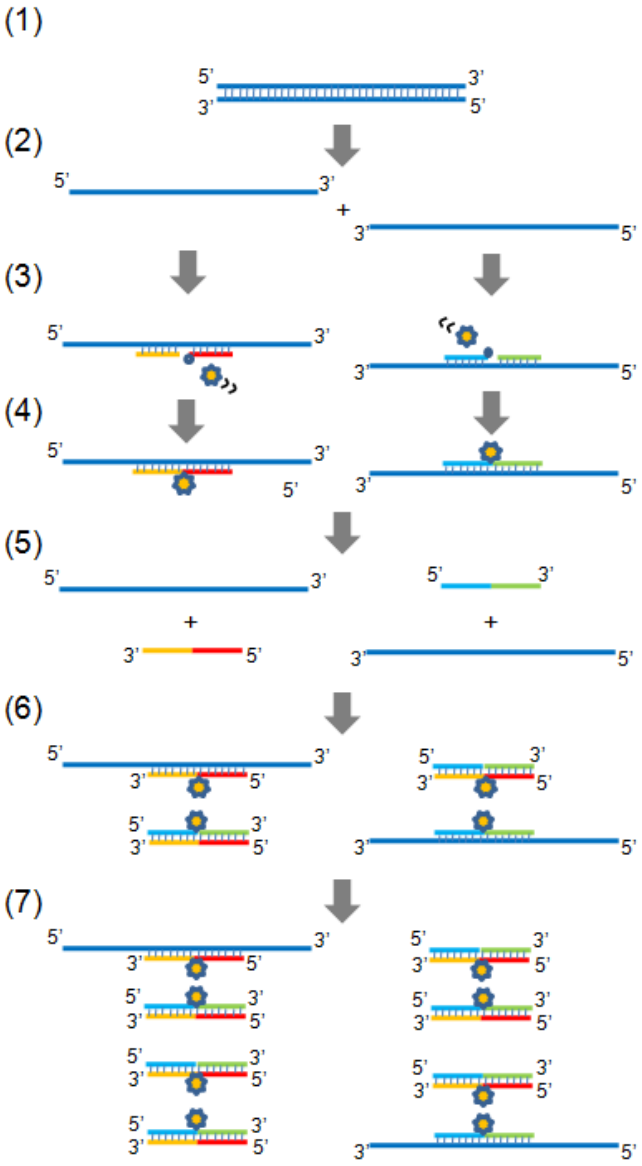

Fig. 2.1. Schematic representation of DNA amplification in ligase chain reaction. (1), Target DNA templates shown as blue bars form double strand in the reaction mixture; (2), DNA templates are denatured at above 90˚C; (3), Oligonucleotide probes shown as red, yellow, blue and green bars are annealed with their target region accompanying with thermostable DNA ligase at a certain temperature about 50-70˚C; (4), The tandem probes are connected about 60˚C; (5), The connected products in the first cycle are denatured at above 90˚C in the second cycle; (6), The probes are annealed and ligated in the second thermal cycle; (7), In the third cycle, the second thermal cycle products are doubled. As the thermal cycles progress, ligated products are exponentially amplified.

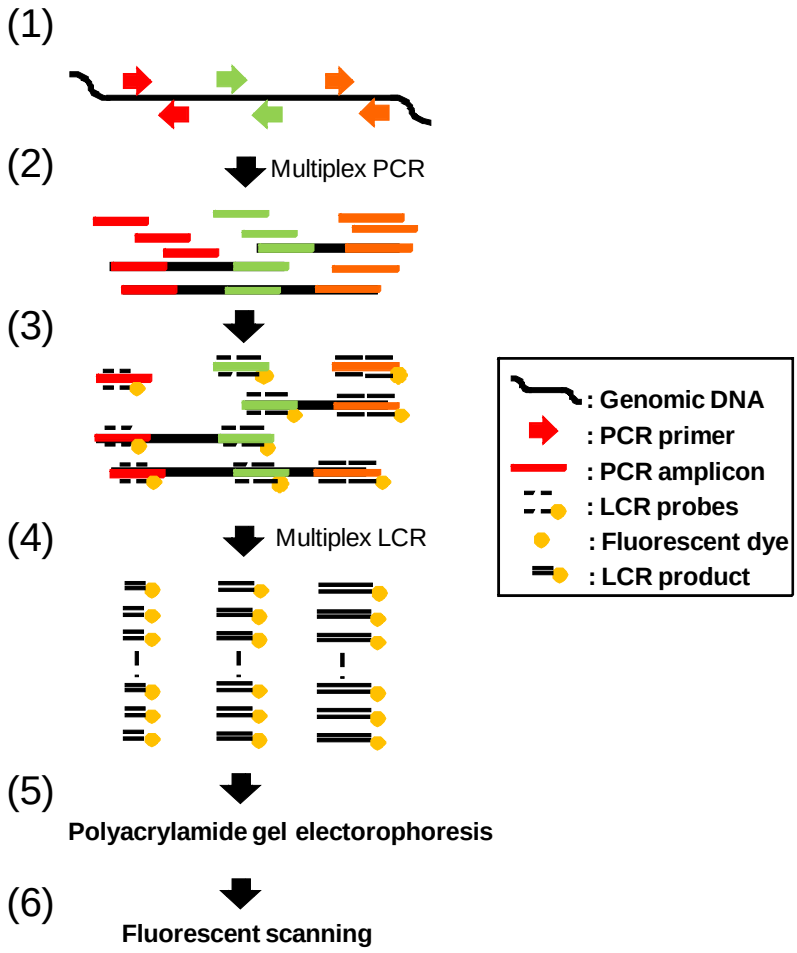

Fig. 2.2. Schematic representation of MPCR-MLCR technique. (1), PCR primers for target DNA regions in a multiplex detection are designed and PCR mixture is prepared; (2), the target DNAs are exponentially amplified by multiplex PCR. In case that the target regions are closely located in a nucleotide chain, intervening DNA regions may also be amplified; (3), LCR probes are designed in the respective target regions of multiplex PCR, and LCR mixture is prepared with the LCR probes labeled with fluorescent dyes; (4), LCR products with different lengths for the respective targets are exponentially amplified by multiplex LCR; (5), polyacrylamide gel electrophoresis is performed and LCR products are separated by the lengths of nucleotide chains; (6), the separated LCR products are detected by fluorescent scanning.

2.2 Materials and methods

2.2.1 Cereal materials

The following representative GM maize events were used: Bt11, Event176, GA21, MON810, MON863, NK603, T25, and TC1507. The representative GM soy event was 40-3-2 (Roundup Ready soybean, RRS). F1 generation seeds of Bt11, Event176 and ground F1 generation seeds of GA21 were kindly provided by Syngenta Seeds AG (Basel, Switzerland). F1 generation seeds of MON810, MON863, and NK603 were kindly provided by MONSANTO Co. (St. Louis, MO, USA). F1 generation seeds of TC1507 were kindly provided by Dow AgroSciences LLC (Indianapolis, IN, USA). F1 generation seeds of T25 and progeny seeds of RRS were directly imported from the USA. Dry maize seeds (Quality Technology International, Inc., Huntley, IL, USA) and dry soybeans harvested in Ohio in 1998 were directly imported and used as non-GM maize and non-GM soy, respectively. Seeds of the conventional rice variety Kinuhikari (*Oryza sativa*), the conventional wheat variety Haruyutaka (*Triticum aestivum*), and the conventional barley variety Harrington (*Hordeum vulgare*) were obtained in Japan.

2.2.2 Preparation of test samples and DNA extraction

Dry seeds were ground with a P-14 speed rotor mill (Fritsch GmbH, Ibar-Oberstein, Germany). The ground materials were stored at -20˚C until DNA extraction. DNA extraction was performed with the DNeasy Plant Maxi kit (Qiagen GmbH, Hilden, Germany) as previously reported (Kuribara et al. 2002). The DNA concentration was determined by measuring ultraviolet (UV) absorbance, and quality was evaluated by the absorbance ratios at 260/280 nm and 260/230 nm. The absorbance ratios at 260/230 nm and 260/280 nm were above 1.7 and between 1.7 and 2.0, respectively. All extracted DNAs were diluted to 20 ng/μl. As a template DNA, simulated maize DNA mixtures and simulated soy DNA mixtures were prepared by mixing genomic DNA (20 ng/μl) from GM flour with genomic DNAs (20 ng/μl)

from non-GM flour at the volume ratio as follows. Simulated maize DNA mixtures, each containing one of the GM maize events, i.e., Bt11, Event176, GA21, MON810, MON863, NK603, T25, or TC1507, at the concentration of 1% (v/v) or 0.2% (v/v), were prepared. Simulated soy DNA mixtures containing a GM soy event, RRS, at the concentration of 1% (v/v) were prepared. Simulated maize DNA mixtures containing all eight GM maize events, i.e., Bt11, Event176, GA21, MON810, MON863, NK603, T25, and TC1507, at the concentrations of 1% (v/v) each, 0.5% (v/v) each, 0.25% (v/v) each, 0.1% (v/v) each, 0.05% (v/v) each, and 0.025% (v/v) each were also prepared. Simulated maize DNA mixtures at the concentration of 99.9% (v/v) MON810 & 0.1% (v/v) GA21, 99.5% (v/v) MON810 & 0.5% (v/v) GA21, 99% (v/v) MON810 & 1% (v/v) GA21, 1% (v/v) MON810 & 99% (v/v) GA21, 0.5% (v/v) MON810 & 99.5% (v/v) GA21, and 0.1% (v/v) MON810 & 99.9% (v/v) GA21 were prepared.

2.2.3 PCR primers and LCR probes

Target regions were selected as follows: a region of phosphinotricin-*N*-acetyltransferase gene derived from *Streptomyces hygroscopicus* (PAT), a region of 5-enolpyruvylshikimate-3-phosphate synthase gene derived from *Agrobacterium* sp. CP-4 (CP4-EPSPS), a region of phosphinotricin-*N*-acetyltransferase gene from *Streptomyces viridochromogenes* (BAR), a region of neomycin phosphotransferase II gene (NPTII), the terminator region of nopaline synthase derived from *Rhizobium radiobacter* (TNOS), the 35S promoter region derived from Cauliflower mosaic virus (P35S) and the promoter region of rice actin gene (PACT). In addition, another MPCR-MLCR system for three endogenous DNA segments was designed as a positive control test. Target regions for this assay were selected as follows: a region of lectin 1 gene of *Glycine max* (Le1), a region of starch synthase IIb gene of *Zea mays* (SSIIb), and the consensus region of 18S ribosomal RNA gene in plant genomes (18SrRNA).

The sequences of PCR primers and their references have been previously reported as listed in Table 2.1 (Kuribara et al., 2002; Matsuoka et al., 2002; Yoshimura et al., 2005) except for 18SrRNA. The oligonucleotide DNAs for PCR primers were synthesized by Fasmac Co., Ltd. (Kanagawa, Japan). The primers, 18SrRNA 3-5' and 18SrRNA 3-3', were designed for the amplification of 18S ribosomal RNA genes as highly conserved nucleotide sequences in crops. For the design of this primer pair, 18S ribosomal RNA gene sequences of some crops, such as *Zea mays* (Genbank Accession No. AF168884), *Hordeum vulgare* (Genbank Accession No. AY552749), *Gossypium hirsutum* (Genbank Accession No. L24145), *Solanum tuberosum* (Genbank Accession No. X67238), *Oryza sativa* (Genbank Accession No. AF069218), *Glycine max* (Genbank Accession No. X02623), *Nicotiana tabacum* (Genbank Accession No. AJ236016), and *Triticum aestivum* (Genbank Accession No. AJ272181), were aligned. The primer pair was designed in a DNA region completely matching in these crops.

For LCR amplification of a target DNA segment, four LCR probes were designed in a region between the forward primer and the reverse primer of each corresponding PCR amplicon. The designed probes and their references are listed in Table 2.2 (Kuribara et al., 2002; Yoshimura et al., 2005; McElroy et al., 1990). Among the four LCR probes, one probe was synthesized with a fluorescent dye, 6-carboxyfluorescein (FAM) at the 5' end, and two probes were synthesized with phosphate at the 5' ends. The length of each LCR product was designed to be different from the others as described in Table 2.2. The oligonucleotide DNAs for LCR probes were synthesized by Fasmac Co., Ltd. The LCR probes for the amplification of PAT, CP4-EPSPS, BAR, and NPTII were designed by referring to the publicly available nucleotide sequences (Table 2.2). The LCR probes for amplification of TNOS, P35S, PACT, Le1, and SSIIb were designed by referring to previous reports (McElroy et al., 1990; Kuribara et al., 2002; Yoshimura et al., 2005), whereas those of 18SrRNA were designed by referring to the nucleotide sequence obtained in the alignment of the nucleotide sequences of 18S ribosomal RNA genes as described above.

The structural information of r-DNA integrated in GM maize and GM soy is compiled in Table 2.3, as taken from safety assessment documents published by the Ministry of Health, Labour and Welfare and the Ministry of Agriculture, Forestry and Fisheries of Japan or documents downloadable from the website of the Japanese Biosafety-Clearing House (http://www.bch.biodic.go.jp/) managed by the Ministry of the Environment of Japan.

Table 2.1. List of PCR primer sequences.

Target name	Primer name	Sequence	Amplicon	Reference
PAT	pat 1-5'	5'-AAGAGTGGATTGATGATCTAGAGAGGT-3'	161 bp	Matsuoka et al., 2002
	pat 1-3'	5'- ATGCCTATGTGACACGTAAACAGTACT-3'		
CP4-EPSPS	epsps 1-5'	5'- GCCTCGTGTCGGAAAACCCT-3'	118 bp	Matsuoka et al., 2002
	epsps 3-3'	5'- TTCGTATCGGAGAGTTCGATCTTC-3'		
BAR	bar 2-5'	5'- ACTGGGCTCCACGCTCTACA-3'	186 bp	Matsuoka et al., 2002
	bar 2-3'	5'- AAACCCACGTCATGCCAGTTC-3'		
NPTII	npt 1-5'	5'- GACAGGTCGGTCTTGACAAAAAG-3'	155 bp	Matsuoka et al., 2002
	npt 1-3'	5'- GAACAAGATGGATTGCACGC-3'		
TNOS	NOS ter 2-5'	5'-GTCTTGCGATGATTATCATATAATTTCTG-3'	151 bp	Kuribara et al., 2002
	NOS ter 2-3'	5'-CGCTATATTTTGTTTTCTATCGCGT-3'		
P35S	P35S 1-5'	5'-ATTGATGTGATATCTCCACTGACGT-3'	101 bp	Kuribara et al., 2002
	P35S 1-3'	5'-CCTCTCCAAATGAAATGAACTTCCT-3'		
PACT	rAct pro 2-5'	5'- CGTTGCAGCGATGGGTAT-3'	121 bp	Matsuoka et al., 2002
	rAct pro 1-3'	5'- GGGCTTGCTATGGATCGTG-3'		
Le1	Le1n02-5'	5'- GCCCTCTACTCCACCCCCA-3'	118 bp	Kuribara et al., 2002
	Le1n02-3'	5'- GCCCATCTGCAAGCCTTTTT-3'		
SSIIb	SSIIb 3-5'	5'- CCAATCCTTTGACATCTGCTCC-3'	114 bp	Yoshimura et al., 2005
	SSIIb 3-3'	5'- GATCAGCTTTGGGTCCGGA-3'		
18SrRNA	18SrRNA 3-5'	5'- CCGTTAACGAACGAGACCTCAGCC-3'	238 bp	This study
	18SrRNA 3-3'	5'- AATGATCTATCCCCATCACGATGAAAT-3'		

Table 2.2. List of LCR probe sequences.

Target name	Probe name	Sequence	Amplicon	Reference
PAT	PAT-1-A	5'-GATAGATACCCTTGGTTGGTTGCTGAGGTTGAGGGTGT-3'	76 bp	Genbank
	Phos-PAT-1-B	5'-phosphate-TGTGGCTGGTATTGCTTACGCTGGGCCCTGGAAGGCTA-3'		DQ156557
	FAM-PAT-1-C	5'-FAM-TAGCCTTCCAGGGCCCAGCGTAAGCAATACCAGCCACA-3'		
	Phos-PAT-1-D	5'-phosphate-ACACCCTCAACCTCAGCAACCAACCAAGGGTATCTATC-3'		
CP4-EPSPS	EPSPS-1-A	5'-AAACCCTGTCACGGTGGACGATGCCACGATGAT-3'	71 bp	US patent
	Phos-EPSPS-1-B	5'- phosphate-CGCCACGAGCTTCCCGGAGTTCATGGACCTGATGGCCG-3'		No. 5804425
	FAM-EPSPS-1-C	5'-FAM-CGGCCATCAGGTCCATGAACTCCGGGAAGCTCGTGGCG-3'		
	Phos-EPSPS-1-D	5'-phosphate-ATCATCGTGGCATCGTCCACCGTGACAGGGTTT-3'		
BAR	BAR-1-A	5'-GGTCGCTGTCATCGGGCTGCCCAACGACCCGAG-3'	66 bp	Genbank
	Phos-BAR-1-B	5'-phosphate-CGTGCGCATGCACGAGGCGCTCGGATATGCCCC-3'		X05822
	FAM-BAR-1-C	5'-FAM-GGGGCATATCCGAGCGCCTCGTGCATGCGCACG-3'		
	Phos-BAR-1-D	5'-phosphate-CTCGGGTCGTTGGGCAGCCCGATGACAGCGACC-3'		
NPTII	NPT-2-A	5'-TCTTGACAAAAAGAACCGGGCGCCCCTGCGCTG-3'	61 bp	Genbank
	Phos-NPT-2-B	5'-phosphate-ACAGCCGGAACACGGCGGCATCAGAGCA-3'		U00004
	FAM-NPT-2-C	5'-FAM-TGCTCTGATGCCGCCGTGTTCCGGCTGT-3'		
	Phos-NPT-2-D	5'-phosphate-CAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGA-3'		
TNOS	TNOS-1-A	5'-GTAATGCATGACGTTATTTATGAGATGG -3'	56 bp	Kuribara et al., 2002
	Phos-TNOS-1-B	5'-phosphate-GTTTTTATGATTAGAGTCCCGCAA -3'		
	FAM-TNOS-1-C	5'-FAM-ATAATTGCGGGACTCTAATCATAAAAAC -3'		
	Phos-TNOS-1-D	5'-phosphate-CCATCTCATAAATAACGTCATGCATTAC-3'		
P35S	P35S-1-A	5'-GACGCACAATCCCACTATCCTTC -3'	51 bp	Kuribara et al., 2002
	Phos-P35S-1-B	5'-phosphate-GCAAGACCCTTCCTCTATATAAGGAAGT-3'		
	FAM-P35S-1-C	5'-FAM-ACTTCCTTATATAGAGGAAGGGTCTTGC -3'		
	Phos-P35S-1-D	5'-phosphate-GAAGGATAGTGGGATTGTGCGTC-3'		
PACT	PACT-2-A	5'-AGACTCAAAACATTTACAAAAAC-3'	46 bp	McElroy et al., 1990
	Phos-PACT-2-B	5'-phosphate-AACCCCTAAAGTTCCTAAAGCCC-3'		
	FAM-PACT-2-C	5'-FAM-GGGCTTTAGGAACTTTAGGGGTT-3'		
	Phos-PACT-2-D	5'-phosphate-GTTTTTGTAAATGTTTTGAGTCT-3'		
Le1	Le1-1-A	5'-GGTGAAGTTGAAGGAAGCGGCGA-3'	51 bp	Kuribara et al., 2002
	Phos-Le1-1-B	5'-phosphate-AGCTGGCAACGCTACCGGTTTCTTTGTC-3'		
	FAM-Le1-1-C	5'-FAM-GACAAAGAAACCGGTAGCGTTGCCAGCT-3'		
	Phos-Le1-1-D	5'-phosphate-TCGCCGCTTCCTTCAACTTCAC-3'		
SSIIb	SSIIb-1-A	5'-AGCAAAGTCAGAGCGCTGCAATG-3'	46 bp	Yoshimura et al.,
	Phos-SSIIb-1-B	5'-phosphate-CAAAACGGAACGAGTGGGGGCAG-3'		
	FAM-SSIIb-1-C	5'-FAM-CTGCCCCCACTCGTTCCGTTTTG-3'		
	Phos-SSIIb-1-D	5'-phosphate-CATTGCAGCGCTCTGACTTTGCT-3'		
18SrRNA	18SrRNA-3-A	5'-CGTGCGGCCCAGAACATC-3'	41 bp	This study
	Phos-18SrRNA-3-B	5'-phosphate-TAAGGGCATCACAGACCTGTTAT-3'		
	FAM-18SrRNA-3-C	5'-FAM-ATAACAGGTCTGTGATGCCCTTA-3'		
	Phos-18SrRNA-3-D	5'-phosphate-GATGTTCTGGGCCGCACG-3'		

2.2.4 Multiplex PCR

For heptaplex (7-plex) PCR designed to amplify seven r-DNA segments, 25 μl of a reaction mixture was assembled containing 50 ng of genomic DNA, 200 μM dNTPs, 0.625 units of AmpliTaq Gold Polymerase (Applied Biosystems, Foster City, CA, USA), 1.5 mM $MgCl_2$, 2.5 μl of 10 × PCR buffer II (Applied Biosystems) and seven primer pairs at the following concentrations: 0.1 μM for PAT, CP4-EPSPS, TNOS, and P35S; 0.14 μM for NPTII; 0.2 μM for PACT; and 0.4 μM for BAR. The concentrations of primer pairs were experimentally adjusted. For triplex (3-plex) PCR designed to amplify three endogenous DNA segments, 25 μl of a reaction mixture was assembled containing the same components as the heptaplex PCR reaction mixture with the exception of three primer pairs at the following concentrations: 0.1 μM for Le1 and SSIIb, and 0.02 μM for 18SrRNA. The PCR amplification was carried out on the ABI PRISM 9700 (Applied Biosystems) with thermal cycles consisting of 95˚C for 10 min for preincubation, 45 cycles of 95˚C for 30 s for denaturation, 55˚C for 30 s for annealing and 72˚C for 30 s for extension, and 72˚C for 7 min for final extension. For the analysis of PCR products, agarose gel electrophoresis was carried out with 3% (w/v) LO3 agarose (Takara Bio Inc., Otsu, Japan) gel in TAE buffer (Wako Pure Chemical Industries, Ltd., Osaka, Japan) with 0.2 μg/ml of ethidium bromide (Sigma Aldrich, St. Louis, MO, USA). Eight microliters of each reaction mixture underwent electrophoresis at a constant voltage (100 V) for 30 min in the TAE buffer. After the electrophoresis, the gel was photographed under UV radiation by using the Densitograph system (ATTO, Tokyo, Japan).

2.2.5 LCR conditions

For simplex LCR, a 10 μl reaction mixture consisting of 1 μl of multiplex PCR mixture as template DNA, 0.1 unit of Ampligase (EPICENTRE Biotechnologies, Madison, WI, USA), 1 μl of the 10 × Reaction Buffer [200 mM Tris/HCl (pH 8.3), 250 mM KCl, 100 mM $MgCl_2$, 5 mM NAD^+, 0.1% of Triton-X], and a set of 4 LCR probes for one target DNA

region with a concentration of 12.5 nM each was used. For the heptaplex LCR which is capable of amplifying seven r-DNA segments, a 10 μl volume of reaction mixture was prepared that was the same as the simplex LCR reaction mixture except that seven sets of the LCR probes listed in Table 2.2 were used for PAT, CP4-EPSPS, BAR, NPT, TNOS, P35S, and PACT as target DNA with a concentration of 12.5 nM each. For the triplex LCR that can amplify three endogenous DNA segments, a reaction mixture was prepared that was the same as the simplex LCR reaction mixture except that three sets of LCR probes (Table 2.2) were used for Le1, SSIIb, and 18SrRNA with a concentration of 12.5 nM each. The LCR amplification was carried out on the ABI PRISM 9700 with thermal cycles consisting of 2 cycles of 94˚C for 1.5 min and 55˚C for 6 min, and 15 cycles of 91˚C for 30 s and 55˚C for 6 min.

2.2.6 Analysis of LCR products

For analysis of LCR products, polyacrylamide gel electrophoresis was carried out on polyacrylamide gel (10%T and 2.6%C) (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in TBE buffer. Six μl of LCR reaction solution mixed with 1.5 μl of a sample loading buffer (6 × Loading buffer triple dye, Nippon Gene Co., Ltd., Tokyo, Japan) was applied along with a DNA size marker, Fluorescein MapMarker 1000 (BioVentures, Inc., Murfreesboro, TN, USA), and electrophoresed at a constant voltage (300 V) for 60 min in TBE buffer. After the electrophoresis, the gel was scanned with the Molecular Imager FX (Bio-Rad Laboratories) connected to 488 nm external laser in the FAM detection mode (medium range). The image data were analyzed with the analytical software Quantity One Version 4.5.2 (Bio-Rad Laboratories).

2.3 Results and discussion

2.3.1 Simplex and multiplex PCR

The specificity of simplex PCR amplification with the PCR primers was confirmed previously (Kuribara et al., 2002; Matsuoka et al., 2002; Yoshimura et al., 2005) except for 18SrRNA. Simplex PCR with the primer pair for 18SrRNA at the concentration of 0.5 μM was performed with genomic DNAs from maize, soy, wheat, barley, and rice as template DNA. DNA amplification was observed on the conventional crop samples of these 5 crops (data not shown). Heptaplex PCR for the seven r-DNA segments (i.e., PAT, CP4-EPSPS, BAR, NPTII, TNOS, P35S, and PACT) and triplex PCR for the three endogenous DNA segments (i.e., Le1, SSIIb, and 18SrRNA) were performed. Agarose gel electrophoreses of heptaplex PCR products and triplex PCR products are shown in Fig. 2.3 (A) and (B), respectively. Some amplification products with the expected lengths (Table 2.1) were observed corresponding to the DNA segments listed in Table 2.3. At the same time, many amplification products with unexpected lengths were observed, and they were possibly attributed to non-specific amplification with unpaired primers. Based on the results of the multiplex PCRs, it could not be determine whether GM crops were included or what kind of DNA segments were contained in each template DNA. This result showed the necessity of the following multiplex LCR.

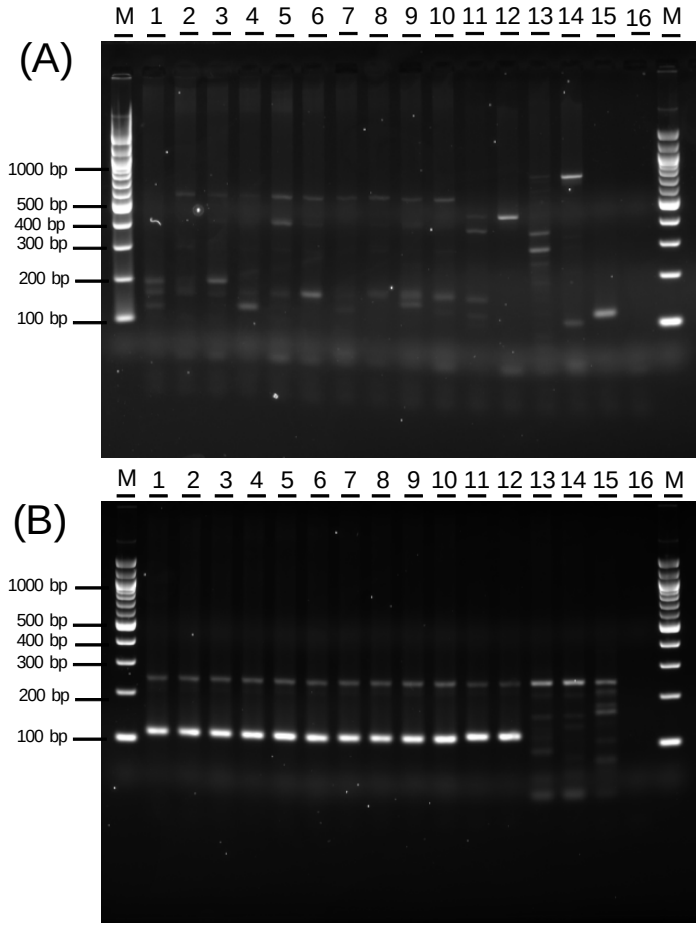

Fig. 2.3. Results of agarose gel electrophoresis of multiplex PCR products. Heptaplex PCR with various kinds of template DNA was performed and electrophoresed (A). Triplex PCR with various template DNA was performed and electrophoresed (B). The template DNA in the multiplex PCR reaction mixture for each lane was as follows: lane 1, the simulated maize DNA mixture containing all eight GM maize events at the concentration of 1% (v/v) each; lanes 2-9, the simulated maize DNA containing each of the GM maize events (Bt11, Event176, GA21, MON810, MON863, NK603, T25, or TC1507, respectively) at the concentration of 1% (v/v); lane 10, non-GM maize genome; lane 11, the simulated soy DNA containing RRS at the concentration of 1% (v/v); lane 12, non-GM soy genome; lanes 13-15, non-GM wheat, non-GM barley, and non-GM rice genome, respectively; lane 16, sterilized distilled water (negative control); lane M, 100 bp ladder size standard.

Table 2.3. Detection targets in GM events of maize and soy.

Type of detection	Target name	Sample name										
		Bt11	E176	GA21	M810	M863	NK603	T25	TC1507	Non-GM maize	RRS	Non-GM soy
r-DNA segment	PAT	+	-	-	-	-	-	+	+	-	-	-
	CP4-EPSPS	-	-	-	-	-	+	-	-	-	+	-
	BAR	-	+	-	-	-	-	-	-	-	-	-
	NPTII	-	-	-	-	+	-	-	-	-	-	-
	TNOS	+	-	+	-	+	+	-	-	-	+	-
	P35S	+	+	-	+	+	+	+	+	-	+	-
	PACT	-	-	+	-	-	+	-	-	-	-	-
Endogenous reference gene	Le1	-	-	-	-	-	-	-	-	-	+	+
	SSIIb	+	+	+	+	+	+	+	+	+	-	-
	18SrRNA	+	+	+	+	+	+	+	+	+	+	+

2.3.2 Multiplex PCR-simplex LCR

Simplex LCR amplification following multiplex PCR was performed as shown in Fig. 2.4. On the odd-numbered lanes of both (A) and (B), LCR amplification was not observed because of a lack of template DNA, and only unreacted LCR probes were found as bands. On the even-numbered lanes, LCR-amplified products with the expected lengths were observed along with concomitantly decreasing LCR probes. It was confirmed that LCR amplification proceeded successively with PCR products as template DNA. In this investigation, multiplex PCR products without purification were added into LCR reaction mixtures as template DNA. Any adverse effects caused by the addition of PCR reaction mixture were not observed in the results of LCR, suggesting that the purification of PCR products was not necessary in our method.

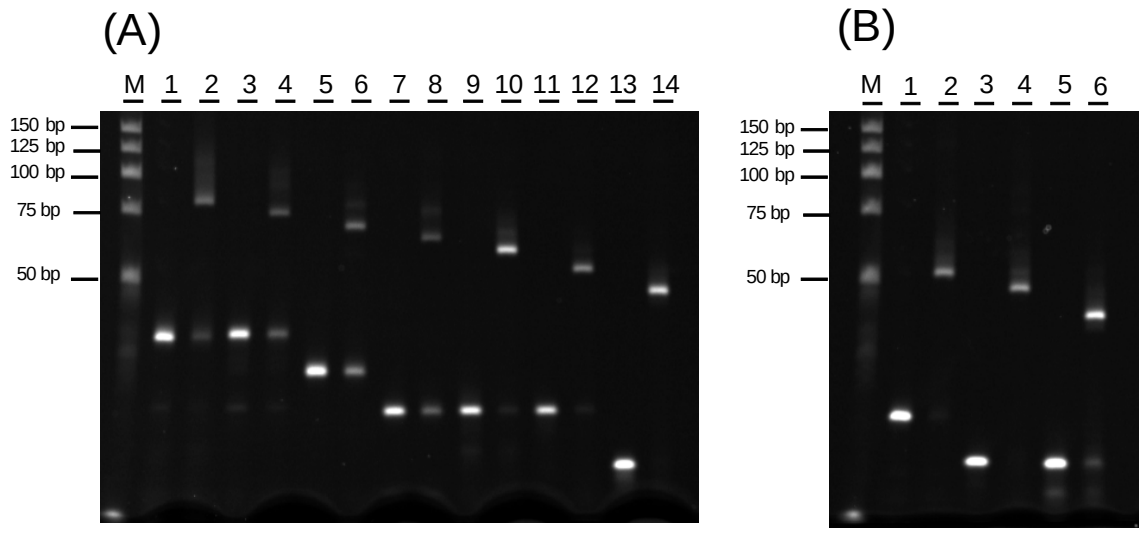

Fig. 2.4. DNA amplification in multiplex PCR-simplex LCR. Simplex LCR was performed with heptaplex PCR products as template DNA, and polyacrylamide gel electrophoresis was carried out (A). LCR for amplification of the following segments was used: lanes 1-2, PAT; lanes 3-4, CP4-EPSPS; lanes 5-6, BAR; lanes 7-8, NPTII; lanes 9-10, TNOS; lanes 11-12, P35S; lanes 13-14, PACT. In odd-numbered lanes, LCR was carried out with PCR reaction products with no template DNA. In even-numbered lanes, LCR was performed with PCR products amplified from the simulated maize genomic DNA containing all eight GM maize events at the concentration of 1% (v/v) each. Polyacrylamide gel electrophoresis was carried out with simplex LCR products amplified with triplex PCR products as template DNA (B). LCR for the amplification of the following targets was performed: lanes 1-2, Le1; lanes 3-4, SSIIb; lanes 5-6, 18SrRNA. Triplex PCR products amplified from no template DNA were used as template DNA in LCR in odd-numbered lanes, triplex PCR products amplified from non-GM soy genome were used as template DNA for LCR in lane 2, and triplex PCR products amplified from non-GM maize genome were used as template DNA for LCR in lanes 4 and 6. In Lane M, the DNA size marker was electrophoresed.

2.3.3 Multiplex PCR-multiplex LCR (MPCR-MLCR)

MPCR-MLCR was performed, and the specificity was examined as shown in Fig. 2.5. The LCR amplification observed in each lane corresponded to the information of the detection targets listed in Table 2.3. Regarding non-GM maize, soy, wheat, and barley, no amplification of r-DNA segments was observed as expected. In the r-DNA segment assay with template DNA from non-GM rice (Fig. 2.5 (A), lane 15), only one band indicating the PACT segment was observed. This amplification was also expected because PACT is a DNA segment originally derived from the conventional rice. The results of endogenous reference gene-detection were shown to be specific for the relevant crops (Fig. 2.5 (B)). 18SrRNA was detected for the various crop samples. Thus, the 18SrRNA detection test as a positive control may expand the applicability of this proposal detection method for a broader range of plant species.

The sensitivity of the assay for r-DNA segments was tested with a simulated maize DNA mixture with each GM event at the concentration of 0.2% (v/v). The expected amplification was clearly observed in the same way as Fig. 2.5 (A) (data not shown). In addition, the assay with simulated maize DNA mixtures containing all eight GM maize events with several concentrations was carried out for the simultaneous amplification of seven segments (Fig. 2.6 (A) and (B)). Even when a simulated maize DNA mixture with eight GM events at the concentration of 0.05% (v/v) each was used as template DNA, seven LCR products were clearly observed as expected. The results indicated that the amplification in the multiplex system was well-performed, even with the low concentration of target DNA segments. In this experiment, several segments were included redundantly in the reaction mixture as template DNA. For example, P35S was found in genomic DNA of seven GM events and TNOS was also found in four GM events (Table 2.3). Therefore, it was additionally noted that the copy numbers of each DNA segment were not equal in the simulated maize DNA mixtures which consisted of all eight GM maize events at the same

concentration for each line. Furthermore, the detection sensitivity for a r-DNA segment when another target segment dominantly coexisted in a sample was evaluated (Fig. 2.6 (C) and (D)). The detection of P35S segment derived from MON810 and the detection of TNOS and PACT segments derived from GA21 were selected as representative target segments. In lane 10-12 of Fig. 2.6 (C), the detection of TNOS and PACT under the dominant P35S amplification was evaluated. When the sample contained 0.5% (v/v) GA21 in MON810 genome, all the expected amplification was observed, including weak signal of the PACT amplification. Meanwhile, the sample contained 0.1% (v/v) GA21 in MON810 genome, the PACT amplification was not observed. For the detection of P35S with the dominant TNOS and PACT amplification, all the expected amplification was detected even when the sample contained 0.1% (v/v) MON810 in GA21 as shown in Fig. 2.6 (C). In Fig. 2.6 (D), the endogenous reference genes were detected as expected. From these results, it was supposed that the sensitive detection of underrepresented target segments under the coexistence of other dominant target would be achievable by our method.

In this chapter, the MPCR-MLCR technique was proposed as a new approach to the simultaneous detection of r-DNA segments in GM crops. For the design of this new technique, the author emphasized the high sensitivity and specificity achieved by multiplex PCR and multiplex LCR, respectively. The results of our investigation proved that MPCR-MLCR was efficient, and the availability of multiplex PCR products without purification as template DNA in multiplex LCR made the method simple and less contaminative. Combined PCR-ligase detection reaction-universal array (PCR-LDR-UA) methods have been reported as the first report on the GM crop-detection method with using ligase reaction (Bordoni et al., 2004; Bordoni et al., 2005; Peano et al., 2005). The PCR-LDR-UA aimed at GM event-specific detection with microarray technique, while our investigation aimed at r-DNA segment-detection with electrophoresis-based assay. In the PCR-LDR-UA strategy, the target DNA regions were exponentially amplified only in the

PCR step, and a successful multiplex PCR was supposed to be essential for the sensitive and robust detection. Meanwhile, MPCR-MLCR strategy is mainly based on the multiplex LCR technique and it permits the easy design of multiplex PCR without considering the lengths of the multiple amplification products, because multiplex PCR functions just as pre-amplification. Additionally, the strategy featuring multiplex LCR may render our developed methods more reliable and potentially applicable even to the adjacently located targets. Thus, it is expected that the novel systems for r-DNA segments and endogenous reference genes would be utilized for the efficient screening of GM crops, including unapproved/unknown ones into which any one of the seven r-DNA segments has been introduced, and for the profiling of the segments.

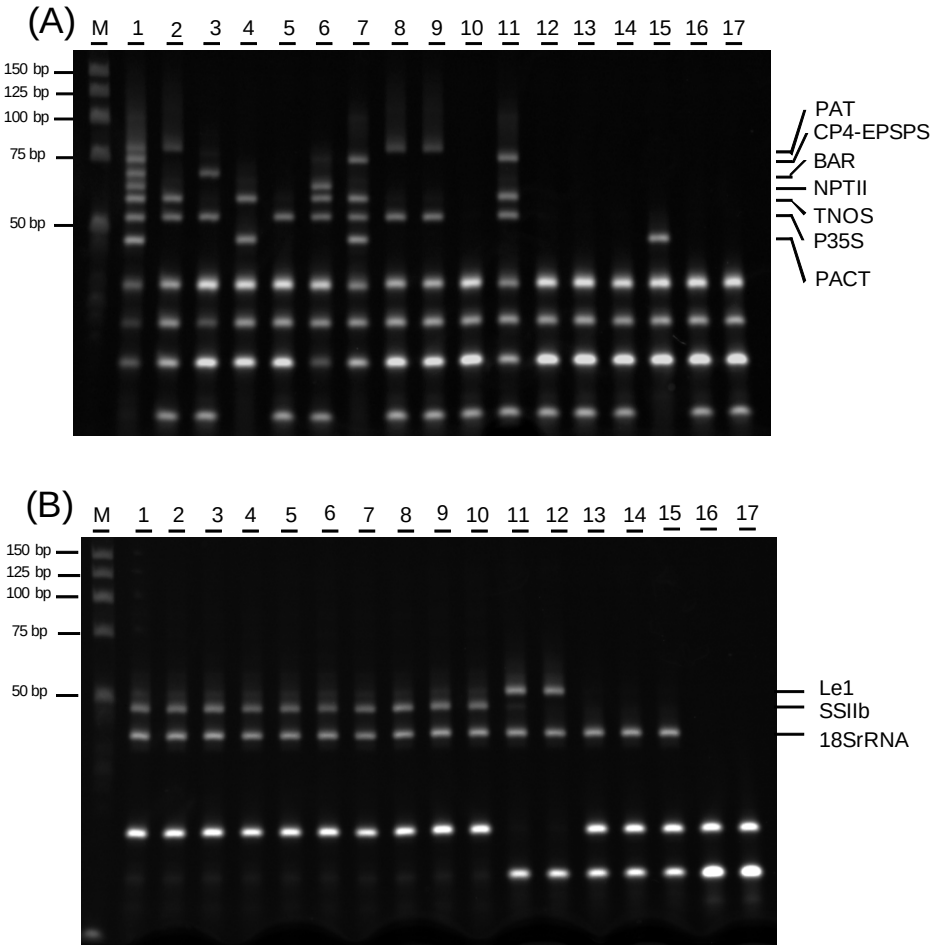

Fig. 2.5. Specificity of MPCR-MLCR detection. The results of polyacrylamide gel electrophoreses with multiplex LCR products for r-DNA segment detection are shown in (A), and those for endogenous DNA segment detection are shown in (B). The MPCR-MLCR products with the following template DNA in multiplex PCR were electrophoresed: lane 1, the simulated maize DNA containing all eight GM maize events at the concentration of 1% (v/v) each; lanes 2-9, the simulated maize DNA containing each of the GM maize events (Bt11, E176, GA21, M810, M863, NK603, T25, or TC1507) at the concentration of 1% (v/v); lane 10, non-GM maize genome; lane 11, the simulated soy DNA containing RRS at the concentration of 1% (v/v); lane 12, non-GM soy genome; lane 13, non-GM wheat genome; lane 14 non-GM barley genome; lane 15, non-GM rice genome; lane 16, sterilized distilled water (negative control in multiplex PCR). In lane 17, multiplex LCR products with sterilized distilled water as template DNA for LCR were electrophoresed as negative controls in multiplex LCR. In lane M, the DNA size marker was electrophoresed.

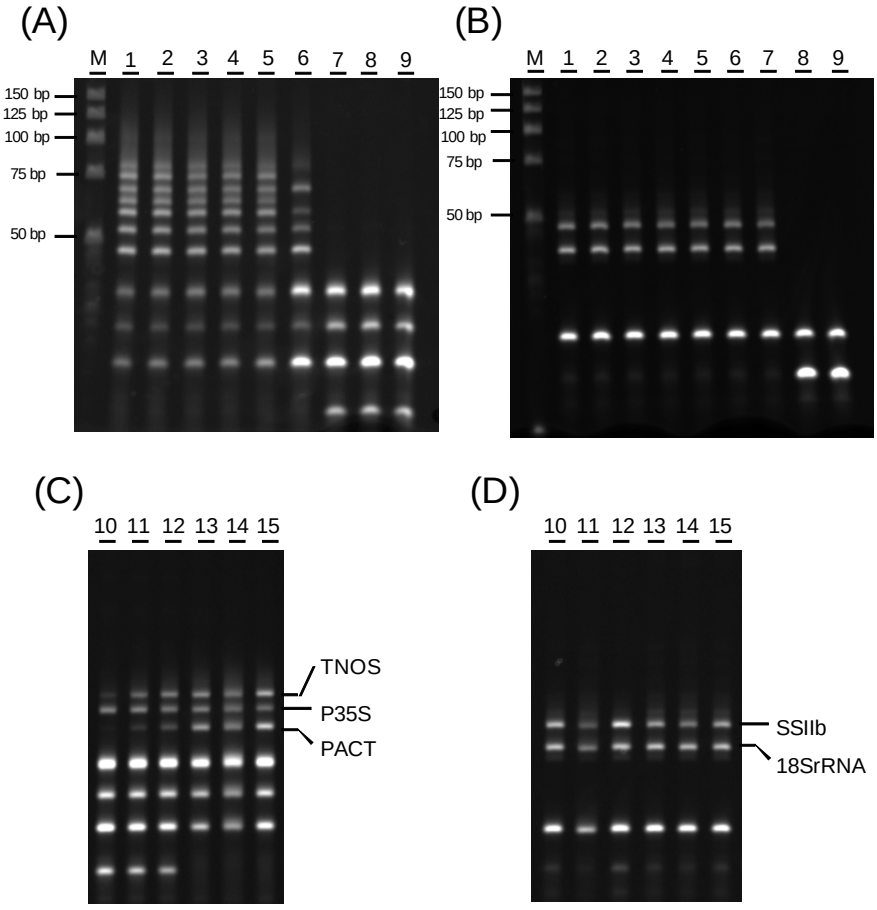

Fig. 2.6. Sensitivity of MPCR-MLCR detection. The results of polyacrylamide gel electrophoresis with reaction mixtures for r-DNA segment detection are shown in (A) and (C), and those with reaction mixtures for endogenous segment detection are shown in (B) and (D). The MPCR-MLCR products with the following genomic DNAs as template DNA in multiplex PCR were electrophoresed: lanes 1-6, the simulated maize DNA mixture containing all eight GM maize events at the concentrations of 1% (v/v) each, 0.5% (v/v) each, 0.25% (v/v) each, 0.1% (v/v) each, 0.05% (v/v) each, and 0.025% (v/v) each, respectively; lane 7, non-GM maize genome; lane 8, sterilized distilled water (negative control); lane 10-15, the simulated maize DNA mixtures at the concentration of 99.9% (v/v) MON810 & 0.1% (v/v) GA21, 99.5% (v/v) MON810 & 0.5% (v/v) GA21, 99% (v/v) MON810 & 1% (v/v) GA21 , 1% (v/v) MON810 & 99% (v/v) GA21, 0.5% (v/v) MON810 & 99.5% (v/v) GA21, and 0.1% (v/v) MON810 & 99.9% (v/v) GA21, respectively. In lane 9, multiplex LCR products with sterilized distilled water as template DNA were electrophoresed as negative controls. In lane M, the DNA size marker was electrophoresed.

Chapter 3

Real-time PCR array as a universal detection platform for genetically modified crops and its application in identifying unapproved genetically modified crops in Japan

3.1 Introduction

Today many types of genetically modified organisms (GMOs), including microorganisms, animals and plants, are already in practical use, and the number of commercially available genetically modified (GM) crops is increasing rapidly (James, 2008). In Japan, a total of 76 events of GM crops have been approved for open field cultivation or provision as food, feed or ornamental plants as living modified organisms (LMOs) under the Japanese Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of LMOs that came into effect in Japan on July 8, 2009. Additionally, a total of 98 GM crop events have been approved for food under the Food Sanitation Law of April 30, 2009. Under these circumstances, it is desirable to develop GM crop testing methods that are capable of collecting a lot of information at once. Simultaneous detection methods, such as multiplex polymerase chain reaction (PCR) methods (Matsuoka et al., 2000; Matsuoka et al., 2001; James et al., 2003; Onishi et al., 2005), DNA chip analysis (Rudi et al., 2003; Bordoni et al., 2004; Bordoni et al., 2005; Peano et al., 2005; Germini et al., 2005; Xu et al., 2005; Xu et al., 2006; Leimanis et al., 2006; Scmidt et al., 2008) and membrane hybridization methods (Su et al., 2003), have been developed and reported for some GM crops. Although multiplex PCR is one of the most efficient and easiest techniques for multiplex detection, the multiplex reaction is difficult to be applied in practical testing and false-positive amplifications tends to occur than in simplex reaction (Markoulatos, 2002; Rudi et al., 2003; Schmidt et al., 2008). In addition, the interaction between individual reactions in the multiplex system causes unstable testing results in cases in which there is a big gap between the copy numbers of the target DNAs (Elnifro et al., 2000; Ratcliff et al., 2007). In the development of an analytical method for regulatory use with GMOs, a validation study among participating laboratories is required to evaluate the performance. Validation studies, however, tend to be time- and cost-consuming. The addition of a single individual reaction into a validated multiplex reaction system may require substantial effort to

re-evaluate the whole system. This makes it difficult to supply suitable GM testing methods to testing laboratories in a flexible and impromptu manner so that they will be ready to deal with the increasing number of approved GM crops. Given this situation, a universal detection system that permits the simultaneous implementation of many individual validated methods would be an efficient and useful tool for GM analysis. The costs of DNA analysis depend largely on the high price of PCR instruments and reagents. The frequent use of an instrument may pay off. Additionally, the large-scale synthesis of oligonucleotides for primers or probes may provide superior cost performance to small-scale synthesis. Thus, a universal detection platform with which many analyses can be performed in a single system is also attractive from an economic point of view. Real-time PCR with TaqMan chemistry has been used in various kinds of quantitative detection methods for GM crops (Kuribara et al., 2002; Holst-Jensen et al., 2003). Furthermore, the validation studies on the detection methods have been reported on the website, "Community Reference Laboratory for GM Food and Feed" (http://gmo-crl.jrc.it/default.htm) under the European Commission. TaqMan PCR provides higher specificity than conventional PCR due to the chemistry with TaqMan probes. Additionally, TaqMan assay development with Applied Biosystems-system does not necessarily require strict optimization of reaction conditions such as thermal cycling or the composition of the reaction buffer with referring to the manufacturer's protocol of TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA). These characteristics are advantageous for a universal detection platform. The ideal system would be easily updatable and customizable as the situation demands, particularly for the addition of new approved GM events.

In Japan, the Food Sanitation Law and the Feed Safety Law require safety assessments of GM crop events for food and feed, respectively. The Japanese Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of LMOs requires the assessment of adverse effects on biological

diversity. Additionally, these laws impose a policy of strict restriction of unapproved GM crops. Nevertheless, the incidents of contamination by unapproved GM events have occurred sporadically and have caused considerable concern worldwide. However, no perfect detection system for all unapproved GM crops has yet been developed because there are no biological or chemical characteristics specific to such crops.

In this chapter, the author proposed real-time PCR array with TaqMan® chemistry, i.e., 96-well PCR plate prepared with a different primer-probe in each well, as a universal platform of GM detection and evaluated the specificity and sensitivity of the developed system. The author also explored the possibility of adopting the real-time PCR array for the control of unapproved GM crops. Additionally, in order to facilitate the assumption of GM crop contamination, the author designed a Microsoft Excel spreadsheet application, Unapproved GMO Checker version 2.01, and made it available on the internet.

3.2 Materials and methods

3.2.1 Cereal materials and DNA extracts for analytical samples

The following GM maize events were used for experiment as the representatives: Bt11, Event176 (E176), GA21, MON810 (M810), MON863 (M863), NK603, T25, TC1507, MIR604, DAS-59122 (D59122), and MON88017 (M88017). Representative GM soy events were the following: 40-3-2 (Roundup Ready Soybean, RRS), A2704-12 (A2704), and A5547-127(A5547). RT73 was used as a representative GM canola event, and LLRICE62 as a representative GM rice event. F1 generation seeds of Bt11 and E176, and ground F1 generation seeds of GA21 were kindly provided by Syngenta Seeds AG (Basel, Switzerland); F1 generation seeds of M810, M863 and NK603 were kindly provided by Monsanto Company (St. Louis, MO, USA); and F1 generation seeds of TC1507 were kindly provided by Dow AgroSciences LLC (Indianapolis, IN, USA). Two certified reference materials (CRMs) in powder form were purchased from the Institute for Reference Materials and

Measurements (IRMM; Retieseweg, Belgium): MIR604 (cat. #, ERM-BF-423d; certified value, 98.5 g/kg; uncertainty (coverage factor k = 2), -2.6 and +2.9 g/kg), and D59122 (ERM-BF-424d; 98.7 g/kg; -5.8 and +5.9 g/kg). Several CRMs were purchased from the American Oil Chemists' Society (AOCS; Urbana, IL, USA): non-modified canola seeds (cat. #: AOCS 0304-A; certified value, below 0.5% of Roundup Ready canola), RT73 seeds (AOCS 0304-B; above 991.9 g/kg of Roundup Ready canola), ground seeds of M88017 (AOCS 0406-D; above 990.5 g/kg), DNA extract of LLRICE62 (AOCS 0306-I; above 999.9 ng/μl of GM DNA), DNA extract of A2704 (AOCS 0707-B; above 999.9 ng/μl of GM DNA) and DNA extract of A5547 (AOCS 0707-C; above 999.9 ng/μl of GM DNA). Plant leaves infected by cauliflower mosaic virus (CaMV) (MAFF Nos. 104018, 104019 and 104021) were obtained as genetic resources from Genebank of the National Institute of Agrobiological Sciences (Tsukuba, Japan). F1 generation seeds of T25 and progeny seeds of RRS were imported directly from the USA. Dry seeds of maize (Quality Technology International, Inc., Elgin, IL, USA) and dry soybeans harvested in Ohio in 1998 were also imported directly and used as non-GM maize and non-GM soy, respectively. Seeds of the conventional rice variety Kinuhikari (*Oryza sativa*), the conventional wheat variety Haruyutaka (*Triticum aestivum*), and the conventional barley variety Harrington (*Hordeum vulgare*) were obtained in Japan. Non-GM CRMs of cotton (ERM-BF422a; below 0.5 g/kg for GM cotton 281-24-236 and 3006-210-23), sugar beet (ERM-BF419a; 0 g/kg for GM sugar beet H7-1) and potato (ERM-BF421a; 0% for GM potato EH92-527-1) were purchased from IRMM.

3.2.2 Preparation of test samples and DNA extraction

All dry seeds except canola seeds were ground with a P-14 speed rotor mill (Fritsch GmbH, Idar-Oberstein, Germany); canola seeds and plant leaves infected by CaMV were frozen in liquid nitrogen, and then ground with an SK mill (Tokken, Inc., Chiba, Japan). The ground materials were stored at -20˚C until DNA extraction. For maize, soy, wheat, barley,

rice, cotton and sugar beet, DNA extraction was performed using a DNeasy Plant Maxi Kit (Qiagen GmbH, Hilden, Germany) as previously reported (Kuribara et al., 2002). For ground samples of canola seeds and potatoes, DNA extraction was performed using a GM quicker 2 (Nippon Gene Co., Ltd., Tokyo, Japan) following the manufacturer's instructions. For ground samples of plant leaves, DNA extraction was performed using a DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. To evaluate the sensitivity, mixed samples of GM and non-GM ground materials at different mass fractions were prepared and used as described below. The DNA concentrations of solutions were determined by measuring ultraviolet (UV) absorbance with a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA) and quality was evaluated by the absorbance ratios at 260/280 nm and 260/230 nm. The absorbance ratio at 260/230 nm was above 1.7 and at 260/280 nm was between 1.7 and 2.0 for most DNA extracts. DNA concentration was calculated with 1 optical density unit at 260 nm equal to 50 ng/μl. All extracted DNAs were diluted to 20 ng/μl with sterile distilled water.

3.2.3 Primers and TaqMan probes

The primers and probes used in the present study and the references (Kuribara et al., 2002; Ding et al., 2004; Cankar et al., 2005; Collonnier et al., 2005; Community Reference Laboratory, 2005; Yoshimura et al., 2005; Weng et al., 2005; Community Reference Laboratory, 2007a; Community Reference Laboratory, 2007b) are listed in Table 3.1. The oligonucleotide DNA for PCR primers and TaqMan probes was synthesized by Fasmac Co., Ltd. (Atsugi, Japan) and Applied Biosystems. The probes were labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamin (TAMRA) at the 5' and 3' ends, respectively, except that the CaMV-MGB probe was labeled with FAM at the 5' end and with non-fluorescent quencher linked with minor groove binder at the 3' end. For GM event detection, 11 GM maize events (Bt11, E176, GA21, M810, M863, NK603, T25, TC1507,

MIR604, D59122 and M88017) and 3 GM soy events (RRS, A2704 and A5547) were selected as targets. Primer-probe set information for the specific detection of M863, NK603 and TC1507 were obtained through personal communication with Kodama *et al.* The author designed primer-probe sets for the detection of M88017 and A5547 by referring to the nucleotide sequence information from international patents WO/2005/059103 and WO/2006/108675, respectively. For recombinant DNA (r-DNA) segment-specific detection, the author selected 10 target segments commonly introduced into some GM crops approved in Japan: the 35S promoter region derived from CaMV (P35S), the terminator region of the nopaline synthase gene derived from *Rhizobium radiobacter* (TNOS), the 35S promoter region of *Figwort mosaic virus* (PFMV), the intron region of the rice actin 1 gene (AINT), a region of the neomycin phosphotransferase II gene (NPTII), a region of the phosphinothricin-*N*-acetyltransferase gene derived from *Streptomyces hygroscopicus* (PAT), a region of the phosphinothricin-*N*-acetyltransferase gene derived from *Streptomyces viridochromogenes* (BAR), a region of the glyphosate oxidoreductase gene derived from *Ochromobactrum anthropi* strain LBAA (GOX), a region of 5-enolpyruvylshikimate-3-phosphate synthase gene introduced into NK603, M88017 and RRS (EPSPS1), and a region of the gene introduced into RT73 (EPSPS2). With respect to the detection of PFMV and AINT, the primer and probe sets were designed by referring to the nucleotide sequence information from GenBank (Accession Nos. NC003554 and X63830, respectively). For NPTII, PAT, BAR and GOX detection, the previously reported primers (Matsuoka et al., 2002) were adopted and probes were designed between the primers by referring to the nucleotide sequence information from GenBank (Accession Nos. U00004, DQ156557, X05822 and AR016595, respectively). The primers for EPSPS1 have been previously reported (Matsuoka et al., 2002) and the probe was designed by referring to nucleotide sequences (US patent 5633435 SEQ ID No. 2 and GenBank Accession No. AB209952). The primers and probe for EPSPS2 were designed by referring to a nucleotide

sequence (US patent 5633435 SEQ ID No. 9). For endogenous reference gene-specific detection, the following target regions of genes were selected: a region of the starch synthase IIb gene of *Zea mays* (SSIIb), a region of the lectin 1 gene of *Glycine max* (Le1), a region of the high-mobility-group protein I/Y gene of rapeseed (HMG), a region of the sucrose phosphate synthase gene of *Oryza sativa* (SPS), and a region of the 18S rRNA gene common in crop plants (18SrRNA). The 18SrRNA gene sequences of some kinds of crops such as *Zea mays* (GenBank Accession No. AF168884), *Hordeum vulgare* (GenBank Accession No. AY552749), *Gossypium hirsutum* (GenBank Accession No. L24145), *Solanum tuberosum* (GenBank Accession No. X67238), *Oryza sativa* (GenBank Accession No. AF069218), *Glycine max* (GenBank Accession No. X02623), *Nicotiana tabacum* (GenBank Accession No. AJ236016) and *Triticum aestivum* (GenBank Accession No. AJ272181) were aligned and the DNA region that matched completely in these crops was selected for the design of the primers-probe set. For donor organism-specific detection, CaMV was selected as a target.

Table 3.1. Primer and probe sequences.

For GM event-detection

Crop name	Target name	Type of detection	Evaluation	Primer or Probe name		Sequence (5'-3')	Length (base)	Amplicon size (bp)	Reference
Maize	Bt11	Construct-specific	A	Bt11 3-5'	5' primer	AAAAGACCACAACAAGCCGC	20	127	Kuribara et al., 2002
				Bt11 3-3'	3' primer	CAATGCGTTCTCCACCAAGTACT	23		
				Bt11-2-Taq	Probe	CGACCATGGACAACAACCCAAACATCA	27		
Maize	E176	Construct-specific	A	E176 2-5'	5' primer	TGTTCACCAGCAGCAACCAG	20	100	Kuribara et al., 2002
				E176 2-3'	3' primer	ACTCCACTTTGTGCAGAACAGATCT	25		
				E176-Taq	Probe	CCGACGTGACCGACTACCACATCGA	25		
Maize	GA21	Construct-specific	A	GA21 3-5'	5' primer	GAAGCCTCGGCAACGTCA	18	133	Kuribara et al., 2002
				GA21 3-3'	3' primer	ATCCGGTTGGAAAGCGACTT	20		
				GA21-2-Taq	Probe	AAGGATCCGGTGCATGGCCG	20		
Maize	M810	Construct-specific	A	M810 2-5'	5' primer	GATGCCTTCTCCCTAGTGTTGA	22	113	Kuribara et al., 2002
				M810 2-3'	3' primer	GGATGCACTCGTTGATGTTTG	21		
				M810-Taq	Probe	AGATACCAAGCGGCCATGGACAACAA	26		
Maize	M863	Event-specific	A	M863 1-5'	5' primer	TGACCCTACTTGTTCGGATGG	21	111	This study
				M863 1-3'	3' primer	GCATTTGTAGGTGCCACCTTC	21		
				MON863-Taq	Probe	CACCCCAAAGTGTACCAAGCTTTCCGA	27		
Maize	NK603	Eventt-specific	A	NK603 1-5'	5' primer	GGCCAGCAAGCCTTGTAGC	19	113	This study
				NK603 1-3'	3' primer	ATCCCGACTCTCTTCTCAAGCATA	24		
				NK603-Taq	Probe	ATGACCTCGAGTAAGCTTGTTAACGCGGC	29		
Maize	T25	Event-Specific	A	PM1	5' primer	TCAATTGCCCTTTGGTCTTCTGA	23	155	Collonier et al., 2005
				revPM1	3' primer	TACGACATGATACTCCTTCCAC	22		
				FBP3	Probe	TCATTGAGTCGTTCCGCCATTGTCG	25		
Maize	TC1507	Construct-specific	A	TC1507 1-5'	5' primer	TGAGTTGATTCCAGTTACTGCCA	23	111	This study
				TC1507 1-3'	3' primer	ATGTTAGTCGCAACGAAACCG	21		
				TC1507-Taq	Probe	ACTCGAGTAAGGATCCGTCGACCTGCAG	28		
Maize	MIR604	Event-specific	B	MIR604 primer F	5' primer	GCGCACGCAATTCAACAG	18	76	Community Reference Laboratory, 2007a
				MIR604 primer R	3' primer	GGTCATAACGTGACTCCCTTAATTCT	26		
				MIR604 probe	Probe	AGGCGGGAAACGACAATCTGATCATG	26		
Maize	D59122	Event-specific	B	DAS59122-7-rb1f	5' primer	GGGATAAGCAAGTAAAAGCGCTC	23	86	Community Reference Laboratory, 2005
				DAS59122-7-rb1r	3' primer	CCTTAATTCTCCGCTCATGATCAG	24		
				DAS59122-7-rb1s probe	Probe	TTTAAACTGAAGGCGGGAAACGACAA	26		
Maize	M88017	Event-specific	B	M88017 1-5'	5' primer	ATCGTGTGACAACGCTAGCA	20	150	This study
				M88017 1-3'	3' primer	CATATTGACCATCATACTCATTGCT	25		
				M88017-1-Taq	Probe	TGCCGGAGTATGACGGTGACGATATATTCA	30		
Soy	RRS	Construct-specific	A	RRS 01-5'	5' primer	CCTTTAGGATTTCAGCATCAGTGG	24	121	Kuribara et al., 2002
				RRS 01-3'	3' primer	GACTTGTCGCCGGGAATG	18		
				RRS-Taq	Probe	CGCAACCGCCCGCAAATCC	19		
Soy	A2704	Event-specific	B	KVM175	5' primer	GCAAAAAAGCGGTTAGCTCCT	21	64	Community Reference Laboratory, 2007b
				SMO001	3' primer	ATTCAGGCTGCGCAACTGTT	20		
				TM031	Probe	CGGTCCTCCGATCGCCCTTCC	21		
Soy	A5547	Event-specific	B	A5547 1-5'	5' primer	CATCGCTATTTGGTGGCATT	20	114	This study
				A5547 1-3'	3' primer	GAATTATGCAGTGCTGCCATAAC	23		
				A5547-1-Taq	Probe	CGCAATGTCATACCGTCATCGTTGTCAG	28		

Table 3.1. Continued.

For r-DNA segment detection

Crop name	Target name	Type of detection	Evaluation	Primer or Probe name		Sequence (5'-3')	Length (base)	Amplicon size (bp)	Reference
Consensus	P35S	r-DNA segment-specific	A	P35S 1-5'	5' primer	ATTGATGTGATATCTCCACTGACGT	25	101	Kuribara et al., 2002
				P35S 1-3'	3' primer	CCTCTCCAAATGAAATGAACTTCCT	25		
				P35S-Taq	Probe	CCCACTATCCTTCGCAAGACCCTTCCT	27		
Consensus	TNOS	r-DNA segment-specific	A	NOS ter 2-5'	5' primer	GTCTTGCGATGATTATCATATAATTTCTG	29	151	Kuribara et al., 2002
				NOS ter 2-3'	3' primer	CGCTATATTTTGTTTTCTATCGCGT	25		
				NOS-Taq	Probe	AGATGGGTTTTTATGATTAGAGTCCCGCAA	30		
Consensus	PFMV	r-DNA segment-specific	A	PFMV 1-5'	5' primer	ATCAACAAGGTACGAGCCATATC	23	120	This study
				PFMV 1-3'	3' primer	TGAGGCTTTGGACTGAGAATTC	22		
				PFMV-1-Taq	Probe	CCAAGAAGGAACTCCCATCCTCAAAGGTTT	30		
Consensus	AINT	r-DNA segment-specific	B	AINT 2-5'	5' primer	TCGTCAGGCTTAGATGTGCTAGA	23	112	This study
				AINT 2-3'	3' primer	CTGCATTTGTCACAAATCATGAA	23		
				AINT-2-Taq	Probe	TTTGTGGGTAGAATTTGAATCCCTCAGC	28		
Consensus	NPTII	r-DNA segment-specific	A	npt 1-5'	5' primer	GACAGGTCGGTCTTGACAAAAAG	23	155	This study
				npt 1-3'	3' primer	GAACAAGATGGATTGCACGC	20		
				NPT-1-Taq	Probe	CCCTGCGCTGACAGCCGGA	19		
Consensus	PAT	r-DNA segment-specific	A	pat 1-5'	5' primer	AAGAGTGGATTGATGATCTAGAGAGGT	27	161	This study
				pat 1-3'	3' primer	ATGCCTATGTGACACGTAAACAGTACT	27		
				PAT-1-Taq	Probe	TGCTTACGCTGGGCCCTGGAAG	22		
Consensus	BAR	r-DNA segment-specific	A	bar 2-5'	5' primer	ACTGGGCTCCACGCTCTACA	20	186	This study
				bar 2-3'	3' primer	AAACCCACGTCATGCCAGTTC	21		
				BAR-1-Taq	Probe	CATGCTGCGGGCGGCCGGCTTCAAGCACGG	30		
Consensus	GOX	r-DNA segment-specific	B	gox 2-5'	5' primer	TGCCAGGAAACTTGACTAGCG	21	103	This study
				gox 2-3'	3' primer	CGAATCAACCAAGGCATGATG	21		
				GOX-1-Taq	Probe	TCCAAAGTGGCTTCTTGACCCAATGG	26		
Consensus	EPSPS1	r-DNA segment-specific	A	epsps 1-5'	5' primer	GCCTCGTGTCGGAAAACCCT	20	118	This study
				epsps 3-3'	3' primer	TTCGTATCGGAGAGTTCGATCTTC	24		
				EPSPS-1-Taq	Probe	TGGACGATGCCACGATGATCGC	22		
Consensus	EPSPS2	r-DNA segment-specific	B	EPSPS2 1-5'	5' primer	GTCTCGTTTCTGAAAACCCTGT	22	118	This study
				EPSPS2 1-3'	3' primer	TTAGTGTCGGAGAGTTCGATCTTAG	25		
				EPSPS2-1-Taq	Probe	TGATCGCTACTAGCTTCCCAGAGTTCATGG	30		

For endogenous reference gene-detection (positive control)

Crop neme	Target name	Type of detection	Evaluation	Primer or Probe name		Sequence (5'-3')	Length	Amplicon (bp)	Reference
Maize	SSIIb	Endogenous reference gene-specific	A	SSIIb 3-5'	5' primer	CCAATCCTTTGACATCTGCTCC	22	114	Yoshimura et al., 2005
				SSIIb 3-3'	3' primer	GATCAGCTTTGGGTCCGGA	19		
				SSIIb-Taq	Probe	AGCAAAGTCAGAGCGCTGCAA	21		
Soy	Le1	Endogenous reference gene-specific	A	Le1n02-5'	5' primer	GCCCTCTACTCCACCCCCA	19	118	Kuribara et al., 2002
				Le1n02-3'	3' primer	GCCCATCTGCAAGCCTTTTT	20		
				Le1-Taq	Probe	AGCTTCGCCGCTTCCTTCAACTTCAC	26		
Rice	SPS	Endogenous reference gene-specific	A	SPSF	5' primer	TTGCGCCTGAACGGATAT	18	81	Ding et al., 2004
				SPSR	3' primer	CGGTTGATCTTTTCGGGATG	20		
				SPSP	Probe	GACGCACGGACGGCTCGGA	19		
Canola	HMG	Endogenous reference gene-specific	A	hmg-F	5' primer	GGTCGTCCTCCTAAGGCGAAAG	22	99	Weng et al., 2005
				hmg-R	3' primer	CTTCTTCGGCGGTCGTCCAC	20		
				hmg-P	Probe	CGGAGCCACTCGGTGCCGCAACTT	24		
Consensus	18SrRNA	Endogenous reference gene-specific	A	18SrRNA 2-5'	5' primer	TGTTGGCCTTCGGGATCGGAGTA	23	111	This study
				18SrRNA 2-3'	3' primer	GCTTTCGCAGTTGTTCGTCTTTCA	24		
				18SrRNA-2-Taq	Probe	TCGGGGGCATTCGTATTTCATAGTCAGA	28		

For donor organism detection (negative control)

Donor name	Target name	Type of detection	Evaluation	Primer or Probe name		Sequence (5'-3')	Length	Amplicon (bp)	Reference
CaMV	CaMV	Donor organisms-specific	A	CaMVF	5' primer	GGCCATTACGCCAACGAAT	19	89	Cankar et al., 2005
				CaMVR	3' primer	ATGGGCTGGAGACCCAATTTT	21		
				CaMV-MGB	Probe	TTCTCCGAGCTTTGTC	16		

3.2.4 Preparation of real-time PCR array, reaction conditions and data analysis

For the preparation of the real-time PCR array, 2 μl of a primer and probe mixture containing 2.5 μM primers and 1 μM probe, was added into each well of a 96-well plate and sealed with MicroAmp Optical Adhesive Film (Applied Biosystems). For a negative control test with no primers or probes, sterile distilled water was used in place of the primer and probe mixture. Array plates containing primer and probe mixtures were preserved under -20˚C until just before use. For the assay of sample DNA with the real-time PCR array, the diluted DNA samples described above, TaqMan Universal PCR Master Mix (Applied Biosystems) and sterile distilled water were mixed and added into each well at a volume of 8 μl. Finally, 10 μl of the reaction mixture in each well contained 20 ng of genomic DNA, 5 pmol of 5' primer, 5 pmol of 3' primer, 2 pmol of a probe and 5 μl of TaqMan Universal PCR Master Mix. The plates containing reaction mixtures were sealed with MicroAmp Optical Adhesive Film, thermal cycled with the ABI PRISM 7500 real-time PCR system (Applied Biosystems), and then data analysis was carried out using Sequence Detection Software Version 1.4 (Applied Biosystems). The thermal cycling conditions were as follows: 2 min at 50˚C, 10 min at 95˚C, 45 cycles of 15 s at 95˚C and 1 min at 60˚C under 9600 emulation mode. Data analysis was performed using the "Amplification Plot" feature of the analysis software and the detail settings were set at the "Delta Rn vs. Cycle" view with Manual Ct mode (Threshold, 0.256) and Manual baseline mode (start of baseline, 3; end of baseline, 10). Amplification lines crossed with the threshold line were determined as positive.

3.2.5 Evaluation of specificity

To evaluate the specificity, DNA extraction was performed twice from each ground sample and each DNA sample was analyzed 3 times. For A2704, A5547 and LLRICE62, two parallel dilutions of CRM samples were performed and the resultant samples were analyzed 3 times each. Specificity was confirmed when the 6 sets of PCR data corresponded to the

relevant information. The A- and B-groups of the primer-probe sets listed in Table 3.1 were evaluated separately.

3.2.6 Evaluation of sensitivity

To evaluate the sensitivity, simulated test samples containing GM crops at several concentrations were prepared. Ground GM crop samples were mixed in ground non-GM crop samples at different mass fractions and the following samples were prepared: 0.1% Bt11, 0.1% E176, 0.1% M863, 0.1% each of 8-event mixtures of GM maize (Bt11, E176, GA21, M810, M863, NK603, T25 and TC1507), 0.25% Bt11, 0.25% E176, 0.25% the 8-event mixture of GM maize, and 0.25% RT73. The ground RRS sample was mixed with the ground non-GM soy sample at a mass fraction of 0.1%. For MIR604, D59122 and M88017, two parallel DNA extractions and dilutions were performed and the samples were mixed with non-GM maize DNA samples (20 ng/μl) at a concentration of 0.25%. For A2704 and A5547, two parallel dilutions of CRMs were performed and the resultant samples were then mixed with non-GM soy DNA samples (20 ng/μl) at a concentration of 0.25%. For LLRICE62, two parallel dilutions of CRMs were performed and the resultant samples were mixed with non-GM rice DNA samples (20 ng/μl) at a concentration of 0.25%. Two DNA solutions for every simulated sample were analyzed 5 times each. The A- and B- groups of the primer-probe sets listed in Table 3.1 were evaluated separately. To evaluate the sensitivity of CaMV detection, DNA extracts were prepared from ground plant leaves infected with the virus (MAFF No. 104019) and they were then analyzed in order to calculate the copy numbers of P35S derived from CaMV by quantitative analysis of the P35S region using the standard method for the detection of GM crops in Japan, as detailed in “The Genetically Modified Food Test and Analysis Manual for Individual Products” by the Food and Agricultural Materials Inspection Center (The Food and Agricultural materials Inspection center, 2002; Moriuchi et al., 2007). Diluted samples containing the CaMV genome at

concentrations of 10^4, 10^3, 10^2 and 10 copies were prepared and assayed with real-time PCR array. Two dilution samples for each concentration were prepared and each was analyzed 5 times.

3.2.7 Evaluation of linearity of amplification in semiquantitative analyses

Linearity with respect to Cycle threshold (Ct) values and the copy number of target DNAs was evaluated. Detections for Bt11, E176, GA21, M810, P35S, TNOS and SSIIb were selected as a representative detection system in the real-time PCR array. Five concentrations (8, 50, 600, 8000 and 100,000 copies per reaction) of control plasmids (Kuribara et al., 2002) containing the nucleotide sequences of the above-mentioned 7 targets were purchased from Nippon Gene Co. Ltd.

3.2.8 Development of the spreadsheet application, Unapproved GMO Checker

Microsoft Excel 2007 was used to construct a spreadsheet application, Unapproved GMO Checker version 2.01. The worksheet is shown in Fig. 3.1. As an input form for an experimental result, the crop name of the analytical sample was designed to be selectable from “Maize,” “Soy” or “Rice” and checkboxes were prepared to input the qualitative results of the real-time PCR array. The “Check” button is linked with a macro that integrates experimental results in the input form and estimates the possibility of unapproved GM crop contamination. The three columns were created, i.e., “Validity of the experimental result,” “Comment about approved GMO” and “Comment about unapproved GMO,” for the output of verification results and a checkbox for the output of r-DNA segments in the unapproved GMO. The logics in the macro were designed so that the validity of experimental results would be confirmed first. For this purpose, the checker examines the correspondence of all of the relationships between the selected crop name and the results of endogenous reference gene-specific detection, and those between the selected crop name and the results of GM

event detection. Additionally, it checks whether all the expected r-DNA segments elicited from the results of GM event detection were detected. If all of these conditions are satisfied, the message “Reasonable” appears as the output in the first column, “Validity of the experimental result,” and the macro continues to the next step in the process. If the conditions are not satisfied, the message “Unreasonable” appears as the output in the first column, “Verification was impossible” appears below “Comment about approved GMO” and “Comment about unapproved GMO” and the macro would be finished. Next, contamination of an approved GMO would be examined based on the results of GM event detection. If approved GM crops are detected, the message “Approved GMO was detected” appears under “Comment about approved GMO;” if not, “Approved GMO was not detected” appears. Finally, unapproved GM contamination would be examined. For this purpose, the expected r-DNA segments deduced based on the results of GM event detection would be compared with the results of r-DNA segment detection. If r-DNA segments other than the expected r-DNA segments are detected, contamination by unapproved GM crops would be suspected, the message “Possible contamination by an unapproved GMO” would appear under “Comment about unapproved GMO,” and the segments expected to be contained in the unapproved GM crop would be shown in the checkbox below “Recombinant segments in the unapproved GMO.”

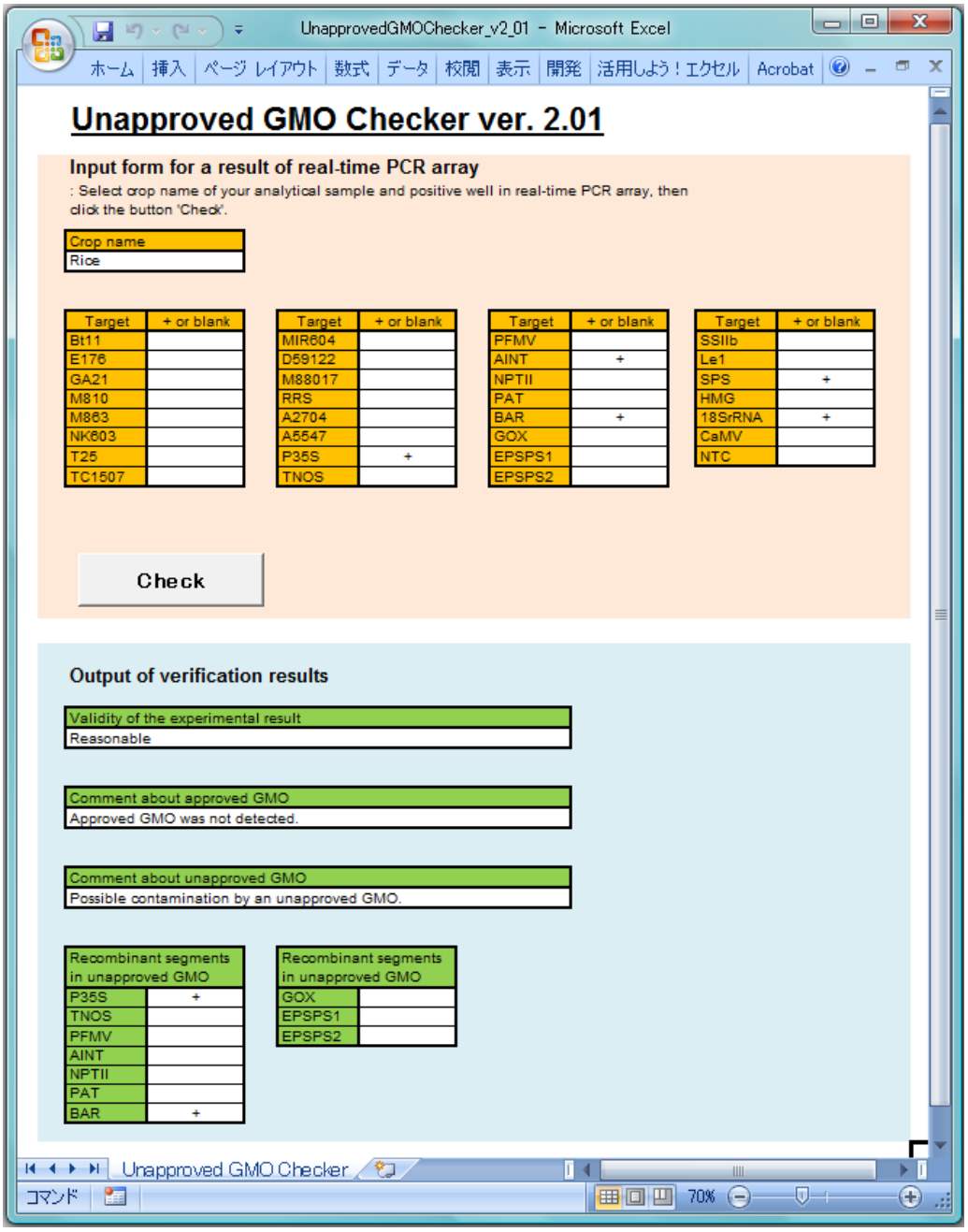

Fig. 3.1. The spreadsheet application, Unapproved GMO Checker version 2.01.

3.3 Results and discussion

3.3.1 Design of the real-time PCR array

The real-time PCR array was designed for the comprehensive detection of GM crops. In the present investigation, a total of 30 primer-probe sets were prepared for GM event detection including event-specific and construct-specific detections as previously reported (Holst-Jensen et al., 2003), r-DNA segment detection, endogenous reference gene detection as a positive control test, and donor organism detection as a negative control test. GM event-detection primer-probe sets were designed aiming for the efficient identification of approved GM maize and soy events. Meanwhile, all 76 events of LMOs approved for open-field cultivation or provision as food, feed or ornamental plants under the Japanese Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of LMOs were expected to contain at least one target of the r-DNA segment detections based on the published information. The r-DNAs common in many GM crop events were confirmed to be amplified and r-DNA segment specific detection was found to be effective for the detection of an extremely broad range of GM crop events. The present system is expected to be a useful tool for screening GM crops regardless of GM events and would be helpful to strictly distinguish GM and non-GM seeds for the practical coexistence of GM crop farming and conventional farming.

The reaction conditions were designed by referring to Japanese standard methods and the validated European methods according to the website of "Community Reference Laboratory for GM Food and Feed" (http://gmo-crl.jrc.it/statusofdoss.htm). For cost reduction, the volume of the reaction mixtures was set at 10 μl, which is the smallest volume recommended by the manufacturer of the real-time PCR apparatus. The total experimental work for one assay including the preparation of the PCR mixtures, thermal cycling and data analysis took only 3 hours. Representative assay results of the real-time PCR array with 31 targets containing 30 designed reactions and a negative control reaction with no primer-probe

set are shown in Fig. 3.2. The following detections were clearly determined to be positive: 18SrRNA, SSIIb, Bt11, P35S, TNOS and PAT for Bt11 maize; 18SrRNA and SSIIb for non-GM maize; 18SrRNA, SPS, P35S, AINT and BAR for LLRICE62; 18SrRNA, AINT and SPS for non-GM rice. These results are consistent with the publicly available information regarding GM crops. The present real-time PCR array successfully distinguished GM and non-GM crops and provided information regarding the GM events and the r-DNA segments by a simple assay.

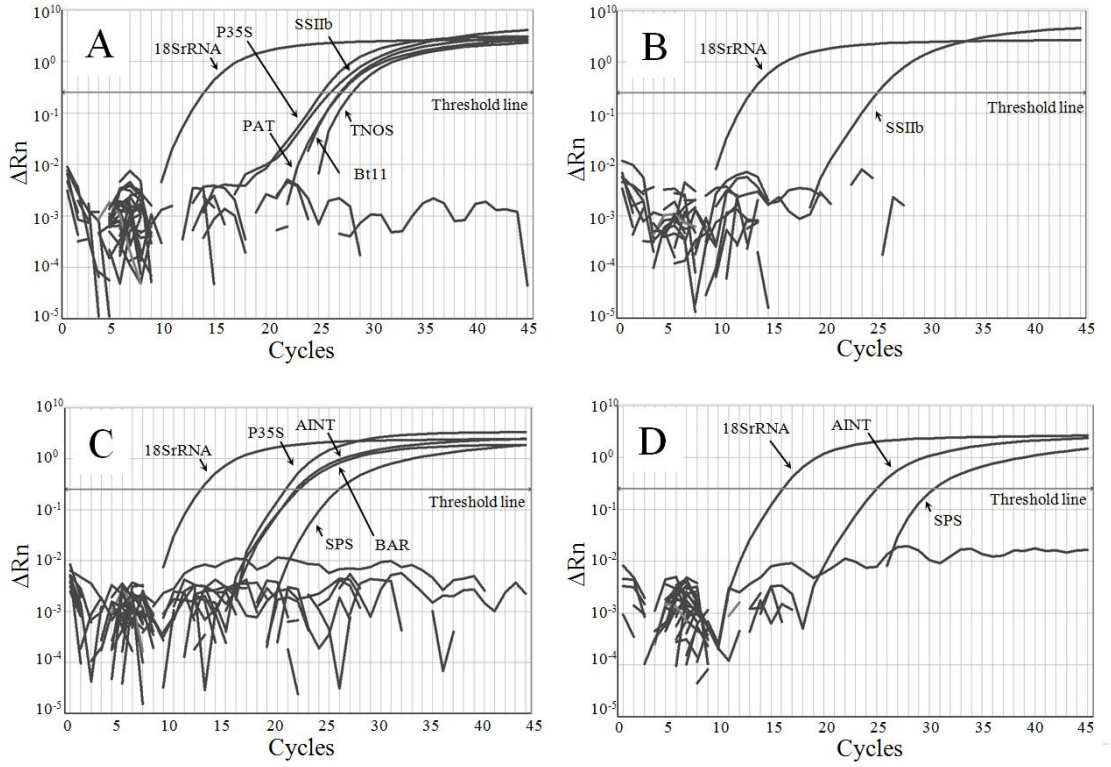

Fig. 3.2. Representative amplification curves in real-time PCR array results. The horizontal axis indicates the cycle numbers of PCR and the vertical axis indicates the ΔRn values, which are the relative values automatically calculated by the analysis software based on signal intensities of FAM dye dependent on the target amplification and ROX passive reference dye. The DNA samples derived from Bt11 (A), non-GM maize (B), LLRICE62 (C) and non-GM rice (D) were assayed for 31 targets as described in the Materials and Methods section. The arrows with target names indicate corresponding amplification curves which were determined to be positive.

3.3.2 Evaluation of specificity

To evaluate the specificity, the samples were prepared and 6 assays were performed. None of the qualitative results presented in Table 3.2 showed any discrepancy with the expected results based on the r-DNA information published from Agbios website (http://www.agbios.com/main.php). In addition to the results shown in Table 2, non-GM wheat, barley, cotton, sugar beet and potato samples were assayed, and only 18SrRNA was positive as predicted. Throughout the specificity evaluation, no non-specific amplification attributed to the inappropriate design of primers or probes was observed and the detection system was found to be applicable to a broad range of crops including maize, soy, rice, canola, wheat, barley, cotton, sugar beet and potato. With respect to CaMV detection, the DNA samples extracted from 3 types of CaMV-infected plant leaves were assayed and specific amplification was identified. In addition to the CaMV detection, the P35S, HMG and 18SrRNA detections were also positive for all infected leaf samples. The detection of P35S was attributed to the genome of infected CaMV, and the detection of HMG and 18SrRNA was caused by plant leaves of *Brassica napus* or a closely related species.

Table 3.2. Results of specificity evaluation.

Type of detection	Target name	Sample name																				
		Bt11	E176	GA21	M810	M863	NK603	T25	TC1507	MIR604	D59122	M88017	Non-GM maize	RRS	A2704	A5547	Non-GM soy	LLRICE 62	Non-GM rice	RT73	Non-GM canola
GM event detection	Bt11	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	E176	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	GA21	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	M810	-	-	-	+		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	M863	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	NK603	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	T25	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	TC1507	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	MIR604	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	D59122	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
	M88017	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	RRS	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
	A2704	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	A5547	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
r-DNA segment detection	P35S	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	-	+	-	-	-
	TNOS	+	-	+	-	+	+	-	-	+	-	+	-	+	-	-	-	-	-	-	-
	PFMV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	AINT	-	-	+	-	+	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-
	NPTII	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PAT	+	-	-	-	-	-	+	+	-	+	-	-	-	+	+	-	-	-	-	-
	BAR	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
	GOX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	EPSPS1	-	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-
	EPSPS2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Endogenous reference gene detection	SSIIb	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	Le1	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
	SPS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
	HMG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	18SrRNA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Donor organism detection	CaMV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Negative control	NTC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

3.3.3 Evaluation of sensitivity

The preparation of simulated samples and their 10 assays were performed as indicated in Table 3.3. False negative results were observed in the assay with several simulated samples containing GM crops at a concentration of 0.1%, specifically, 0.1% Bt11, 0.1% E176 and 0.1% the 8 events of GM maize, but not in the assay with the 0.25% GM samples. The false negative results were considered to have been caused by small copy numbers of target DNAs. High sensitivities were observed for GM event and r-DNA segment detections even under the low concentrations of target DNA (Table 3.3, asterisks). Because maize and canola seeds are reproduced through cross pollination while soy and rice generally self-pollinate, some maize and canola seeds has commonly heterozygous genome. Therefore, to evaluate the sensitivity for these crops, it is thought to be better to use heterozygous samples. Since the zygosity of the M88017 and RT73 CRMs purchased from AOCS has not been defined, it has to be note that the copy number of target DNAs in a 0.25% M88017 or 0.25% RT73 sample corresponds to that of a sample made from absolutely heterozygous seeds with a concentration between 0.25% and 0.5%. Concerning CaMV detection, no false negative results were obtained in the 10 assays with diluted DNA samples at a concentration of 100 copies/μl of CaMV DNA. Meanwhile, amplification failed 6 times in the 10 assays with the diluted samples at a concentration of 10 copies/μl of CaMV DNA. These results suggest that the minimum concentration of CaMV DNA for a reliable result is 100 copies/μl. It was speculated that 0.5% of GM crop-contamination in conventional maize, soy, canola or rice would allow us to obtain accurate results in all wells of the real-time PCR array. The present results demonstrate that the sensitivity of our proposed method is sufficient for the inspection of unintended mixing of approved GM crops under Japanese regulations with a threshold of 5%.

Table 3.3. Results of sensitivity evaluation.

Type of Detection	Target name	Sample name										
		A	B	C	D	E	F	G	H	I	J	K
GM event detection	Bt11	0	0	10	0	10*	0	0	0	0	0	0
	E176	0	0	0	10	10*	0	0	0	0	0	0
	GA21	0	0	0	0	10*	0	0	0	0	0	0
	M810	0	0	0	0	10*	0	0	0	0	0	0
	M863	10*	0	0	0	10	0	0	0	0	0	0
	NK603	0	0	0	0	10*	0	0	0	0	0	0
	T25	0	0	0	0	10*	0	0	0	0	0	0
	TC1507	0	0	0	0	10*	0	0	0	0	0	0
	MIR604	0	0	0	0	0	10*	0	0	0	0	0
	D59122	0	0	0	0	0	0	10*	0	0	0	0
	M88017	0	0	0	0	0	0	0	10*	0	0	0
	RRS	0	10*	0	0	0	0	0	0	0	0	0
	A2704	0	0	0	0	0	0	0	0	10*	0	0
	A5547	0	0	0	0	0	0	0	0	10*	0	0
r-DNA segment detection	P35S	10*	10	10	10	10	0	10	10	10	10	0
	TNOS	10*	10	10	0	10	10	0	10	0	0	0
	PFMV	0	0	0	0	0	0	0	0	0	0	10*
	AINT	10*	0	0	0	10	0	0	10	0	10	0
	NPTII	10*	0	0	0	10	0	0	0	0	0	0
	PAT	0	0	10*	0	10	0	10	0	10	0	0
	BAR	0	0	0	10*	10	0	0	0	0	10	0
	GOX	0	0	0	0	0	0	0	0	0	0	10*
	EPSPS1	0	10*	0	0	10	0	0	10	0	0	0
	EPSPS2	0	0	0	0	0	0	0	0	0	0	10*
Endogenous reference gene detection	SSIIb	10	0	10	10	10	10	10	10	0	0	0
	Le1	0	10	0	0	0	0	0	0	10	0	0
	SPS	0	0	0	0	0	0	0	0	0	10	0
	HMG	0	0	0	0	0	0	0	0	0	0	10
	18SrRNA	10	10	10	10	10	10	10	10	10	10	10
Donor organism detection	CaMV	0	0	0	0	0	0	0	0	0	0	0
Negative control	NTC	0	0	0	0	0	0	0	0	0	0	0

Sample A: 0.1% M863; B: 0.1% RRS; C: 0.25% Bt11; D: 0.25% E176;
E: 0.25% the 8 events of GM maize; F: 0.25% MIR604; G: 0.25% D59122;
H: 0.25% M88017; I: 0.25% A2704 and A5547; J: 0.25% LLRICE62; K: 0.25% RT73.

The value of each element indicates the number of positive detections in a total of 10 assays.
Asterisks indicate data used for the evaluation of the sensitivity of individual detections.

3.3.4 Evaluation of linearity of amplification in reactions

The linearity of amplification in several representative detections was evaluated using the control plasmids for the Japanese standard GM analytical method (The Food and Agricultural materials Inspection Center, 2002). The plasmids were tested 6 times and the Ct values derived from amplification data were plotted against the log value of the copy number of control plasmids. The linear regression curves from the means of the Ct values and that of the standard deviation in 6 assays were obtained (Fig. 3.3). It was found that there is a relatively large dispersion of Ct values with a small copy number of plasmids. However, all R^2 coefficient values were above 0.99 and linearity of amplification for the 7 detections was ascertained. In addition, because the shapes of the amplification curves in the reactions other than these 7 reactions indicated in Fig. 3.3 were also similar and alteration of Ct values dependent on GM crop contents was observed (data not shown), all of the reactions were also expected to demonstrate similar linearity of amplification. Furthermore, TaqMan® PCR is generally used for quantitative assays. Therefore, the present system shows potential for semiquantitative use and further investigation is recommended.

3.3.5 Development of the spreadsheet application, Unapproved GMO Checker

The author developed Unapproved GMO Checker version 2.01 as a spreadsheet application for the assumption of unapproved GM crop contamination (Fig. 3.1). In the development of the application, unapproved GM crops were conceptually defined as (Unapproved GM crops) = (All GM crops) – (Approved GM crops). Approved GM crops could be selectively detected using GM event detections, while r-DNA segment detections detected various kinds of GM crops ranging from approved to unapproved. Therefore, the assumption of unapproved GM crop contamination is achieved by comparing the results of r-DNA segment-specific detection with those of GM event detection in the real-time PCR array. In the present investigation, unapproved GM crop events were defined as GM crops

which have not been approved for open-field cultivation or provision as food, feed or ornamental plants under the Japanese Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of LMOs in Japan. LLRICE62 is an unapproved GM crop event. Based on the results obtained by the present real-time PCR array (Fig. 3.2C), the contamination of unapproved GM crop(s) was assumed, as shown in Fig. 3.1. This result was obtained by the detection of P35S and BAR as r-DNA segments. The result demonstrated no discrepancy between the obtained data and publicly available information regarding GM crops. The application is available and downloadable online (http://cse.naro.affrc.go.jp/jmano/index.html).

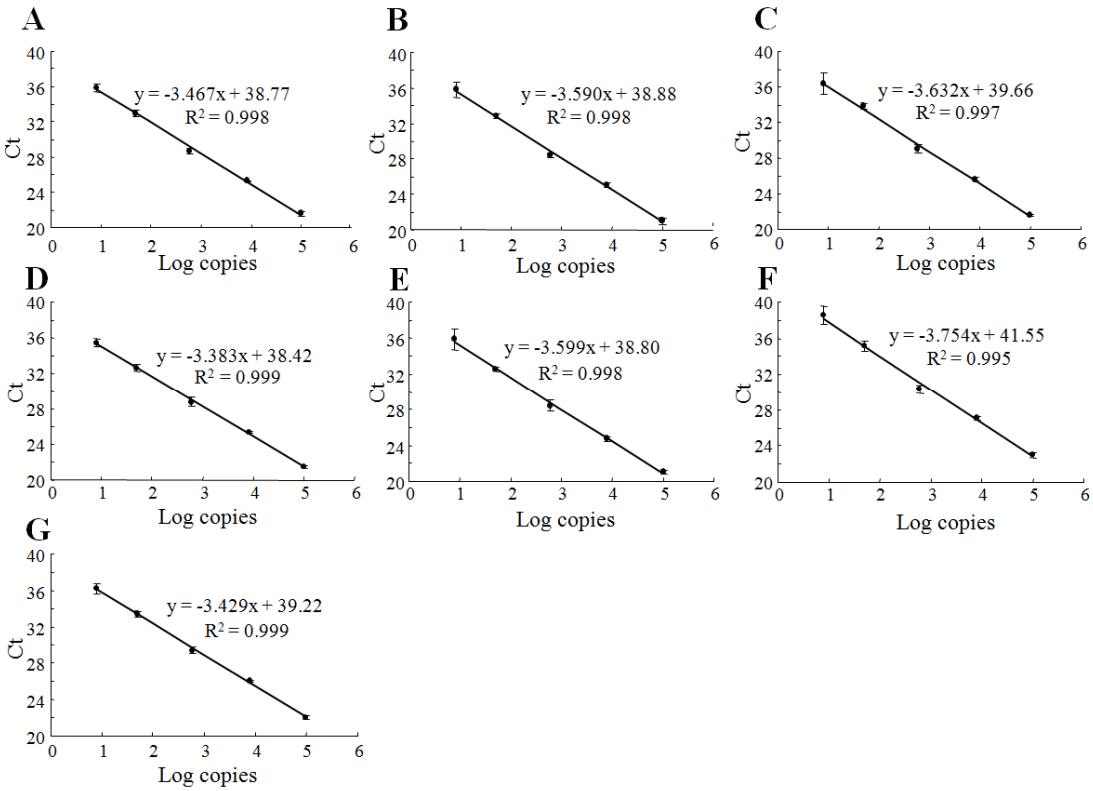

Fig. 3.3. Evaluation of linearity of amplification in real-time PCR array. The calibration plasmids with 5 different copy numbers were assayed as described in the *Materials and Methods* section. The mean Ct values with standard deviations, derived from the amplification data of the reactions listed below, are plotted against the log values of the copy number of the control plasmids. The equation and correlation coefficient value for each linear regression curve are also indicated. A, the Bt11 detection; B, the E176 detection; C, the GA21 detection; D, the M810 detection; E, the P35S detection; F, the TNOS detection; G, the SSIIb detection.

3.3.6 Application of the real-time PCR array to the assumption of unapproved GM crops

For the assumption of unapproved GM crop-contamination, GM maize, GM soy and GM rice were selected as targets. Our assumption could be accomplished only when the appropriate results in all wells of the real-time PCR array were perfectly obtained. The results of the present sensitivity evaluation indicated that a contamination level of 0.5% would be sufficient to obtain reliable data without false negative results. Thus, an analytical sample of fewer than 200 seeds may be preferable. Because unapproved GM crops which have become major concerns such as CBH351 maize, Bt10 maize, LLRICE601, and Bt-rice containing r-DNA segments were selected as target DNA in our investigation, our analytical system may have the potential to discern the novel types of unapproved GM crops as well as the already known unapproved GM crops. However, the present method does not necessarily promise the absolute detection of unapproved GM crops because crops constructed of completely unknown r-DNA segments or r-DNA segments with modified nucleotide sequences cannot be detected. Also, GM event detection does not completely cover all the approved GM crops at present. Furthermore, if approved and unapproved GM crops were mixed in a sample and both crops shared all r-DNA segments, the unapproved GM crop would be masked by the approved GM crop. If unapproved GM crop contamination is suspected, further analysis, such as sequencing of the r-DNA flanking regions, may be required. Despite its many restrictions, the proposed system would serve as an excellent tool to detect unapproved GM contamination. In addition, since the system is able to add new detection sets, it has great potential for expanding its analytical capacity, making use of feed-back information from users about frequently detected approved and unapproved GM events. A differential quantitative PCR technique was recently reported by Cankar *et al.* (Cankar et al., 2008) as a new approach to unapproved/unknown GMO detection. Their method is based on quantitative assay and the accuracy of quantitation with real-time PCR is indispensable. Our

strategy is based on qualitative results and higher reproducibility of the assumption of unapproved GM crop contamination is expected. For low concentrations of GM contents, the present qualitative assay strategy would be advantageous. In addition, the system has potential to detect a much broader range of unapproved GM crops by the various r-DNA segment detections. The utilization of semiquantitative analysis in the present method provides great potential for discovering of unapproved GM crops.

In this chapter,the universal platform for GM crop detection was investigated. It is concluded from the results of the analytical performance-evaluation that the developed real-time PCR array system allows the comprehensive detection of GM crops and the assumption of contamination by unapproved GM crops. This approach is attractive in terms of the specificity of detection, the dynamic range of detection, time efficiency, easy manipulation, updatability and customizability. Another important factor for the dissemination of this new technology is that the proposed method requires no extra investment for equipment in many GMO testing laboratories. Further updating of this system by editing detection targets depending on the purpose of a given investigation would provide appropriate testing methods for both regulatory and commercial use.

Chapter 4

General conclusions

4.1 General conclusions

Due to the rapid increase in the number of approved GM crops in many safety assessment systems, efficient GM crop testing methods have been needed. Many detection techniques enabling simultaneous detection of multiple targets have been investigated. However, the previously developed methods do not have sufficient practical utility. They are particularly inadequate in terms of updatability, in spite of the increasing number of GM events. In this dissertation, the author attempted to construct novel strategies for simultaneous and reliable GM crop detection with high updatability, as well as to establish comprehensive detection methods based on the strategies. Furthermore, the author attempted to apply comprehensive detection methods to manage unapproved GM crops, including unknown ones.

In Chapter 2, the author developed a detection method useful for the efficient screening of GM crops. In this study, a novel strategy, named multiplex PCR-multiplex LCR, MPCR-MLCR, was constructed. It consists of multiplex PCR for preamplification, multiplex LCR with the PCR products as template DNA, polyacrylamide gel electrophoresis and the following fluorescent scanning. In the MPCR-MLCR strategy, multiplex PCR functions as pre-amplification. This enables a multiplex PCR to be easily designed without having to consider the lengths of the multiple amplification products. The strategy featuring multiplex LCR may render our developed methods potentially applicable even to the adjacently located targets. Thus, the MPCR-MLCR strategy has great potential updatability and applicability.

The two MPCR-MLCR systems were designed for multiple r-DNA segments that tend to be introduced into GM crops, and for endogenous reference genes in plant species. The systems that were developed based on this strategy had the high sensitivity and specificity achieved by multiplex PCR and multiplex LCR, respectively. Additionally, the investigation proved that the availability of multiplex PCR products without purification as template DNA in multiplex LCR made the method simple and less contaminative. In addition,

the MPCR-MLCR systems allowed many samples to be analyzed in one experiment by general experimental facilities. The systems can thus be utilized for the high throughput screening of GM crops, including unapproved ones into which any one of the seven r-DNA segments has been introduced. The systems can also be used to profile the segments. For example, the systems can be used to certificate the quality of non-GM seeds for the agriculture in Japan. Many GM crops in new kinds of plants, such as GM creeping bent grass, GM melon, GM plum, GM squash and GM wheat have recently been developed and approved under safety assessment regulations in other countries besides Japan. In these novel GM crops, the r-DNA segments targeted in the present MPCR-MLCR system are frequently introduced and may be detectable. Thus, these new kinds of GM crops classified as unapproved GM crops under the Japanese approval regulations are expected to be managed by using the systems.

One assay with the two MPCR-MLCR systems for the 10 detection targets costs about 1,000 yen without taking into consideration the manpower costs of about 5 hours. Conventional PCR-based testing costs about 100 yen without the manpower costs of 3 hours for one target. Because the manpower costs are estimated at about 1000 yen per hour, the total MPCR-MLCR testing costs are estimated as 6,000 yen as compared to 31,000 yen for conventional PCR testing for the same 10 detection targets. MPCR-MLCR is thus advantageous in terms of time and costs, including manpower costs. Currently, private testing services charge about 20,000 yen per assay to perform screening testing of GM crops. It was thus concluded that the method developed is sufficiently cost effective for commercial use.

In Chapter 3, a universal platform for GM crop detection, which enables exhaustive information on GM crops to be obtained in one assay, was investigated. Real-time PCR array with TaqMan chemistry, i.e., simultaneous implementation of various kinds of TaqMan PCRs targeting for individual GM events, r-DNA segments, endogenous reference genes and donor organisms on a 96-well PCR plate, were adopted as a strategy for universal GM crop

detection. Analytical performances of the thirty reaction components on the array were evaluated, and the reactions showed high sensitivity and specificity for the respective targets. The system developed was proven to achieve comprehensive GM crop detection with high reliability.

Furthermore, the author also explored the possibility of a system of control for unapproved GM crops based on the real-time PCR array strategy. The unapproved GM crops were conceptually defined as (Unapproved GM crops) = (All GM crops) – (Approved GM crops). Approved GM events could be selectively detected using GM event detections, while r-DNA segment detections detected various kinds of GM crops ranging from approved to unapproved. Therefore, the assumption of unapproved GM crop contamination was systematically achieved by comparing the results of r-DNA segment detection with those of GM event detection. In order to facilitate the assumption of GM crop contamination, a Microsoft Excel spreadsheet application, the Unapproved GMO Checker version 2.01, was designed. The real-time PCR array system cannot be guaranteed to provide absolute detection of unapproved GM crops. However, this system would serve as an excellent tool for discovering unapproved GM contamination. In the real-time PCR array system, there are no interactions between individual reactions. The approach is attractive in terms of the specificity of detection, the dynamic range of detection, time efficiency, easy manipulation, updatability and customizability.

In many GM crop inspection systems including the Japanese standard detection methods, the identification of GM events is indispensable. Thus, the present real-time PCR array can be adopted for inspection systems currently used. In addition, detection methods for M863, NK603, T25, TC1507, D59122, M88017 and MIR604 events have not been described in the Japanese standard detection methods and are not detection targets, although a lot of grains include these events. It is thus expected that the present real-time PCR array system will be adopted as a Japanese standard detection method. The knowledge about reaction

components developed on the real-time PCR array can be utilized in the development of real-time PCR-based quantitative detection methods. Continuously updating the real-time PCR array will allow development of an efficient GM crop inspection system that includes screening, identification and quantification of GM crops.

The running cost of the real-time PCR array analysis without the manpower costs is estimated as 10,000 yen for one plate (3 analytes). As described above, conventional PCR-based testing costs about 100 yen without the manpower costs, which are 3 hours for one target. The manpower costs are about 1000 yen per an hour. Thus, the real-time PCR array system costs about 6,300 yen for an analyte including the manpower, while testing by conventional PCR methods cost 93,000 yen for detection of the same 30 target. Private testing services charge 20,000-60,000 yen for a qualitative assay for multiple GM events. The real-time PCR array is also, therefore, cost effective for commercial use.

Recently, the Joint Research Centre under the European Commission developed a comprehensive detection system with a similar concept of the real-time PCR array (Querci et al., 2009). The European system was established upon specific request of the European Parliament in the context of the project "Scientific and technical contribution to the development of an overall health strategy in the area of GMOs." The detection system allows the simultaneous event-specific detections of 39 GMOs, comprising all EU approved and already known unapproved GM events for which a method was submitted to the CRL-GMFF. The Analytical performance of the system, e.g., its specificity and efficiency, were confirmed by experimental testing conducted within the CRL-GMFF, and the system has potential to become one of the EU official methods. The real-time PCR array platform may therefore constitute a significant step towards international harmonization of GMO analyses. If international harmonization of GMO analyses is achieved, then needlessly duplicative inspections in commercial distribution can be avoided (the concept of “one stop testing”). Also from the viewpoint of harmonization, the real-time PCR array is an extremely promising

concept.

Both the MPCR-MLCR and real-time PCR array strategies are applicable for the various GM crop targets and have high updatability. Further updating of these systems by editing detection targets depending on the purpose of a given investigation would provide continuously appropriate testing methods for both regulatory and commercial use. A well-established GM crop control system based on detection methods will guarantee food safety, alleviate consumers' concerns, and conserve the biodiversity of GM crops. Genetic recombination and GM crop cultivation are the best potential technologies for supporting the sustainable development of agriculture needed to feed a growing world population. The author concludes this study have a certain significance in terms of the expansion of possibility that people receive the great benefits of genetic modification technologies.

REFERENCES

Akiyama, H., Watanabe, T., Wakabayashi, K., Nakade, S., Yasui, S., Sakata, K., Chiba, R., Spiegelhalter, F. and Hino, A. and Maitani, T. (2005). Quantitative detection system for maize sample containing combined-trait genetically modified maize. *Analytical Chemistry*, 77, 22, 7421-7428.

Barany, F. (1991a). The ligase chain reaction in a PCR world. *PCR Methods and Applications*, 1, 1, 5-16.

Barany, F. (1991b). Genetic disease detection and DNA amplification using cloned thermostable ligase. *Proceedings of the National Academy of Sciences of the United States of America*, 88, 1, 189-193.

Berg, P., Baltimore, D., Brenner, S., Robin, R. O. and Singer, M. F. (1975). Summary statement of the Asilomar conference on recombinant DNA molecules. *Proceedings of National Academy of Sciences of the United States of America*, 72, 6, 1981-1984.

Berg, P. (2008). Meetings that changed the world: Asilomar 1975: DNA modification secured. *Nature*, 455, 290-291.

Boom, R., Sol, C. J. A., Salimans, M. M. M., Jansen, C. L., Wertheim Van Dillen, P. M. E. and Van der Noordaa, J. (1990). Rapid and simple method for purification of nucleic acid. *Journal of Clinical Microbiology*, 28, 3, 495-503.

Bordoni, R., Mezzelani A., Consolandi, C, Frosini, A., Rizzi, E., Castiglioni B., Salati, C., Marmiroli, N., Marchelli, R., Bernardi, L. R., Battaglia, C. and Bellis, G. D. (2004). Detection and quantitation of genetically modified maize (Bt-176 transgenic maize) by applying ligation detection reaction and universal array technology. *Journal of Agricultural and Food Chemistry*, *52*, 1049-1054.

Bordoni, R., Germini A., Mezzelani, A., Marchelli, R. and Bellis, G. D. (2005), A microarray platform for parallel detection of five transgenic events in foods: a combined polymerase chain reaction-ligation detection reaction-universal array method.

Journal of Agricultural and Food Chemistry, *53*, 912-918.

Butch A. W. (2000). Dilution Protocols for Detection of Hook Effects/Prozone Phenomenon. *Clinical Chemistry, 46,* 1719-1720.

Cankar, K., Ravnikar, M., Zel, J., Gruden, K. and Toplak, N. (2005) Real-time polymerase chain reaction detection of cauliflower mosaic virus to complement the 35S screening assay for genetically modified organisms. *Journal of AOAC International*, 88, 3, 814-822.

Cankar, K., Chauvensy-Ancel, V., Fortabat, M. N., Gruden, K., Kobilinsky, A., Zel, J. and Bertheau, Y. (2008). Detection of nonauthorized genetically modified organisms using differential quantitative polymerase chain reaction: application to 35S in maize. *Analytical Biochemistry*, *376*, 189-199.

Chaouachi, M., El Malki, R., Berard, A., Romaniuk, M., Laval, V., Brunel, D. and Bertheau, Y. (2008a). Development of a real-time PCR method for the differential detection and quantification of four solanaceae in GMO analysis: potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), and pepper (*Capsicum annuum*). *Journal of Agricultural and Food Chemistry*. 56, 6, 1818-1828.

Chaouachi, M., Fortabat, M. N., Geldreich, A., Yot, P., Kerlan, C., Kebdani, N., Audeon, C., Romaniuk, M. and Bertheau, Y. (2008b). An accurate real-time PCR test for the detection and quantification of cauliflower mosaic virus (CaMV): applicable in GMO screening. *European Food Research and Technology*, 227, 789–798.

Cohen, S. N., Chang, A. Y., Boyer, H. W. and Helling, R. B. (1973). Construction of biologically functional bacterial plasmids *in vitro*. *Proceedings of National Academy of Sciences of the United States of America*, 70, 11, 3240-3244.

Collonnier, C., Schattner, A., Berthier, G., Boyer, F., Coué-Philippe, G., Diolez, A., Duplan, M. N., Fernandez, S., Kebdani, N., Kobilinsky, A., Romaniuk, M., de Beuckeleer, M.,

de Loose, M., Windels, P. and Bertheau, Y. (2005). Characterization and event specific-detection by quantitative real-time PCR of T25 maize insert. *Journal of AOAC International*, 88, 2, 536-546.

Community Reference Laboratory for GM Food & Feed. (2007). Event-specific method for the quantification of maize line MIR604 using real-time PCR (protocol). *http://gmo-crl.jrc.it/summaries/MIR604_validated_Method.pdf*.

Community Reference Laboratory for GM Food & Feed. (2005). Event-specific method for the quantification of maize 59122 using real-time PCR (protocol). *http://gmo-crl.jrc.it/summaries/59122-Protocol%20Validation.pdf*.

Community Reference Laboratory for GM Food & Feed. (2007). Event-specific method for the quantification of soybean line A2704-12 using real-time PCR (protocol). *http://gmo-crl.jrc.it/summaries/A2704-12_soybean_validated_Method.pdf*.

Daidenko, V. V. (2001). DNA probes using fluorescence resonance energy transfer (FRET): Designs and Applications. *Biotechniques*, 31, 5, 1106-1121.

Dille, B. J., Butzen, C. C. and Birkenmeyer, L. G. (1993) Amplification of Chlamydia trachomatis DNA by ligase chain reaction. *Journal of Clinical Microbiology*, 31, 3, 729-731.

Ding, J., Jia, J., Yang, L., Wen, H., Zhang, C., Liu, W. and Zhang, D. (2004). Validation of a rice specific gene, sucrose phosphate synthase, used as the endogenous reference gene for qualitative and real-time quantitative PCR detection of transgenes. *Journal of Agricultural and Food Chemistry*, 52, 11, 3372-3377.

Elnifro, E. M., Ashshi, A. M., Cooper, R. J. and Klapper, P. E. (2000). Multiplex PCR: Optimization and application in diagnostic virology. *Clinical Microbiology Reviews*, 13, 4, 559-570.

Engvall, E. and Perlmann, P. (1971). Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immunochemistry*, 8, 871-874.

Freise, J., Gérard, H. C., Bunke, T., Whittum-Hudson, J. A., Zeidler, H., Köhler, L., Hudson, A. P. and Kuipers, J. G. (2001). Optimised sample DNA preparation for detection of Chlamydia trachomatis in synovial tissue by polymerase chain reaction and ligase chain reaction. *Annals of Rheumatic Diseases*, 60, 2, 140-145.

Gašparič, M. B., Cankar K., Žel, J. and Cruden Kristina. (2008). Comparison of different real-time PCR chemistries and their suitability for detection and quantification of genetically modified organisms. *BMC Biotechnology*, 8, 26.

Germini, A., Zanetti, A., Salati, C., Rossi, S., Forré, C., Schmid, S., Marchelli, R. and Fogher, C. (2004). Development of a seven-target multiplex PCR for the simultaneous detection of transgenic soybean and maize in feeds and foods. *Journal of Agricultural and Food Chemistry*, 52, 11, 3275-3280.

Germini, A., Rossi, S., Zanetti, A., Corradini, R., Fogher, C. and Marchelli, R. (2005). Development of a peptide nucleic acid array platform for the detection of genetically modified organisms in food. *Journal of Agricultural and Food Chemistry*, 53, 10, 3958 -3962.

Goda, Y., Asano, T., Shibuya, M., Hino, A. and Toyoda, M. (2001). Detection of recombinant DNA from genetically modified papaya. *Shokuhin Eiseigaku Zasshi*, 42, 4, 231-236 (Japanese).

Grothaus, G. D., Bandla, M., Currier, T., Giroux, R., Jenkins, G. R., Lipp, M., Shan G., Stave, J. W. and Pantella, V. (2006). Immunoassay as an analytical tool in agricultural biotechnology. *Journal of AOAC international*, 89, 4, 913-927.

Heid, C. A., Stevens, J., Livak, K. J. and Williams, P. M. (1996). Real time quantitative PCR. *Genome Methods*, 6, 986-994.

Heide, B. R., Heir, E. and Holck, A. (2008). Detection of eight GMO maize events by qualitative, multiplex PCR and Fuorescence capillary gel electrophoresis. *European*

Food Research and Technology, 227, 527–535.

Hernández, M., Río, A., Esteve, T., Prat, S. and Pla, M. (2001). A rapeseed-specific gene, acetyl-CoA carboxylase, can be used as a reference for qualitative and real-time quantitative PCR detection of transgenes from mixed food samples. *Journal of Agricultural and Food Chemistry*, 49, 8, 3622-3627.

Hernández, M, Rodríguez-Lázaro, D., Esteve, T., Prat, S. and Pla, M. (2003). Development of melting temperature-based SYBR Green I polymerase chain reaction methods for multiplex genetically modified organism detection. *Analytical Biochemistry*, 323, 2, 164-170.

Hino, A. (1999). Zehi shitteokitai idenshi kumikae nousakumotsu, Saiwai shobo.

Höhne, M., Santisi, C. R. and Meyer, R. (2002). Real-time multiplex PCR: An accurate method for the detection and quantification of 35S-CaMV promoter in genetically modified maize-containing food. *European Food Research and Technology*, 215, 59–64.

Holst-Jensen, A., Ronning, S. B., Lovseth, A. and Berdal, K. G. (2003). PCR technology for screening and quantification of genetically modified organisms (GMOs). *Analytical and Bioanalytical Chemistry*, 375, 985-993.

Huang, H. Y. and Pan, T. M. (2004). Detection of Genetically Modified Maize MON810 and NK603 by Multiplex and Real-Time Polymerase Chain Reaction Methods. *Journal of Agricultural and Food Chemistry*, 52, 3264-3268.

Hurst, C. D., Knight, A. and Bruce, I. J. (1999). PCR detection of genetically modified soya and maize in foodstuffs. *Molecular Breeding*, 5, 6, 579-586.

Iida, M., Yamashiro, S., Yamakawa, H., Hayakawa, K., Kuribara, H., Kodama, T., Furui, S., Akiyama, H., Maitani, T. and Hino, A. (2005). Development of taxon-specific

sequences of common wheat for the detection of genetically modified wheat. *Journal of Agricultural and Food Chemistry*, 53, 16, 6294-6300.

James, C. (2008). Executive summary–Global status of Commercialized Biotech/GM Crops: 2008, *ISAAA Briefs* 2008, 39.

James, D., Schmidt A. M., Wall E., Green M. and Masri S. (2003). Reliable detection and identification of genetically modified maize, soybean, and canola by multiplex PCR analysis. *Journal of Agricultural and Food Chemistry*, 24, 5829-5834.

Kato, J. (2005). 2nd section, Atarashii idenshikumikaetaino anzenseihyouka sisutemu gaidobukku, NTS inc.

Kuribara, H., Shindo, Y., Matsuoka, T., Takubo, K., Futo, S., Aoki, N., Hirao, T., Akiyama, H., Goda, Y., Toyoda, M. and Hino, A. (2002). Novel reference molecules for quantitation of genetically modified maize and soybean. *Journal of AOAC International*, 85, 5, 1077-89.

La paz, J. L., García-Muniz, N., Nadal, A., Esteve, T., Puigdoménech, P. and Pla, M. (2006). Interlaboratory transfer of a real-time polymerase chain reaction assay for quantitative detection of genetically modified maize event TC-1507. *Journal of AOAC International*, 89, 5, 1347-52.

Laffler, T. G. and Carrino, J. J. (1993). Marshall, R.L. The ligase chain reaction in DNA-based diagnosis. *Annales de Biologie Clinique*, 51, 9, 821-826.

Leimanis, S., Hernandez, M., Fernandez, S., Boyer, F., Burns, M., Bruderer, S., Glouden, T., Harris, N., Kaeppeli, O., Philipp, P., Pla, M., Puigdomenech, P., Vaitilingom, M., Bertheau, Y. and Remacle, J. (2006). A microarray-based detection system for genetically modified (GM) food ingredients. *Plant Molecular Biology*, *61*, 123-139.

Lee, H. H. (1996). Ligase chain reaction. *Biologicals*, 24, 3, 197-199.

Magaña-Gómez, J. A. and Calderón de la Barca, A. M. (2008). Risk assessment of genetically

modified crops for nutrition and health. *Nutrition Review*, 67, 1, 1-16.

Markoulatos, P., Siafakas, N. and Moncany, M. (2002). Multiplex polemerase chain reaction: a practical approach. *Journal of Clinical Laboratory Analysis*, 16, 1, 47-51.

Matsuoka, T., Kawashima, Y., Akiyama, H., Miura, H., Goda, Y., Sebata, T., Isshiki K., Toyoda, M. and Hino A. (1999). A detection method for recombinant DNA from genetically modified soybeans and processed foods containing them. *Shokuhin Eiseigaku Zasshi*, 40, 2, 149-157. (Japanese).

Matsuoka, T., Kuribara, H., Suefuji S., Miura, H., Kusakabe, Y., Akiyama, H., Goda, Y., Isshiki, K.., Toyoda, M. and Hino A. (2001). A detection method for recombinant DNA from genetically modified maize CBH351. *Shokuhin Eiseigaku Zasshi*, 43, 3, 197-201. (Japanese).

Matsuoka, T., Kuribara, H., Takubo, K., Akiyama, H., Miura, H., Goda Y., Kusakabe, Y., Issiki, K., Toyoda, M. and Hino, A. (2002). Detection of recombinant DNA segments introduced to genetically modified maize (*Zea mays*). *Journal of Agricultural and Food Chemistry*, *50*, 2100-2109.

Matsuoka, T., Kawashima, Y., Akiyama, H., Miura, H., Goda, Y., Kusakabe, Y., Isshiki, K., Toyoda, M. and Hino, A. (2000). A method of detecting recombinant DNAs from four lines of genetically modified maize. *Shokuhin Eiseigaku Zasshi*, 41, 2, 137-143.

Matsuoka, T., Kuribara H., Akiyama, H., Miura, H., Goda, Y., Kusakabe, Y., Isshiki, K., Toyoda, M. and Hino, A. (2001). A multiplex PCR method of detecting recombinant DNAs from five lines of genetically modified maize. *Shokuhin Eiseigaku Zasshi*, 41, 1, 24-32.

Matsuoka, T., Kuribara H., Takubo, K., Akiyama, H., Miura, H., Goda Y., Kusakabe, Y., Issiki, K., Toyoda, M. and Hino, A. (2002). Detection of recombinant DNA segments introduced to genetically modified maize (*Zea mays*). *Journal of Agricultural and*

Food Chemistry, 50, 2100-2109.

Mavropoulou, A. K., Koraki, T., Ioannou, P. C. and Christopoulos, T. K. (2005). High-throughput double quantitative competitive polymerase chain reaction for determination of genetically modified organisms. *Analytical Chemistry*, 77, 15, 4785-4791.

McElroy, D., Zhang. W., Cao, J. and Wu, R. (1990). Isolation of efficient actin promoter for use in rice transformation. *The Plant Cell*, *2*, 163-171.

Mendel, G. (1865). Versuche über Plflanzen-hybriden. Verhandlungen des naturforschenden Ver-eines, 3–47.

Morisset, D., Dobnik, D., Hamels, S., Zel, J. and Gruden, K. (2008). NAIMA: target amplification strategy allowing quantitative on-chip detection of GMOs. *Nucleic Acids Research*, 36, 18, e118.

Moriuchi, R., Monma, K., Sagi, N., Uno, N. and Kamata, K. (2007). Applicability of quantitative PCR to soy processed foods containing Roundup Ready soy. *Food Control*, 18, 191-195.

Murray, M. G. and Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acid Research*, 8, 19, 4321-4325.

Nadal, A., Coll, A., La Paz, J. L., Esteve, T. and Pla, M. (2006). A new PCR-CGE (size and color) method for simultaneous detection of genetically modified maize events. *Electrophoresis*, 27, 19, 3879-3888.

Oguchi, T., Onishi, M., Chikagawa, Y., Minegishi, Y., Kodama, T., Akiyama, H., Ohno, Y., Futo, S., Hino, A., Furui, S. and Kitta, K. (2008). Development of event-specific quantitation method for GA21 maize, which is a GM event without CaMV35S promoter. *Shokuhin Eiseigaku Zasshi*, 49, 1, 16-22.

Onishi, M., Matsuoka T., Kodama, T., Kashiwaba, K., Futo, S., Akiyama H., Maitani, T.,

Furui, S., Oguchi, T. and Hino, A. (2005), Development of a multiplex polymerase chain reaction method for simultaneous detection of eight events of genetically modified maize. *Journal of Agricultural Food Chemistry*, 53, 9713-9721

Ramesaar, K., Capell, T., Twyman, R. M., Quemada, H. and Christou, P. (2008). Trace and traceability –a call for regulatory harmony. *Nature Biotechnology*, 26, 9, 975-978.

Ratcliff, R. M., Chang, G., Kok, T. and Sloots, T. P. (2007). Molecular diagnosis of medical viruses. *Current Issues in Molecular Biology*, 9, 87-102.

Rønning, S. B., Vaïtilingom, M., Berdal, K. G. and Holst-Jensen Arne. (2003). Event specific real-time quantitative PCR for genetically modified Bt11 maize (Zea mays). *European Food Research and Technology*, 216, 347–354.

Rudi, K., Rud, I. and Holck A. (2003). A novel multiplex quantitative DNA array based PCR (MQDA-PCR) for quantification of transgenic maize in food and feed. *Nucleic Acids Research*, 31, 11, e62.

Pan, L., Zhang, S., Yang, L., Broll, H., Tian, F. and Zhang, D. (2007). Interlaboratory trial validation of an event-specific qualitative polymerase chain reaction-based detection method for genetically modified RT73 rapeseed. *Journal of AOAC International*, 90, 6, 1639-46.

Peano, C., Bordoni, R., Gulli, M., Mezzelani, A., Samson, M. C., De Bellis, G. and Marmiroli, N. (2005). Multiplex polymerase chain reaction and ligation detection reaction/universal array technology for traceability of genetically modified organisms in foods. *Analytical Biochemistry*, 346, 90-100.

Permingeat, H. R., Reggiardo, M. I. and Vallejos, R. H. (2002). Detection and Quantification of Transgenes in Grains by Multiplex and Real-Time PCR. *Journal of Agricultural and Food Chemistry*, 50, 4431-4436.

Querci, M., Foti N., Bogni, A., Kluga, L., Broll, H. and Van den Eede. G. (2009). Real-Time PCR-Based Ready-to-Use Multi-Target Analytical System for GMO Detection. *Food Analytical Methods*, 2, 4, 325-336.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, 239, 4839, 487-491.

Schmidt, A. M., Sahota, R., Pope, D. S., Lawrence, T. S., Belton, M. P. and Rott, M. E. (2008). Detection of genetically modified canola using multiplex PCR coupled with oligonucleotide microarray hybridization. *Journal of Agricultural and Food Chemistry*, 56, 16, 6791-6800.

Shimizu, E., Kato, H., Nakagawa, Y., Kodama, T., Futo, S., Minegishi, Y., Watanabe, T., Akiyama, H., Teshima, R., Furui, S., Hino, A. and Kitta, K. (2008). Development of a screening method for genetically modified soybean by plasmid-based quantitative competitive polymerase chain reaction. *Journal of Agricultural and Food Chemistry*. 56, 14, 5521-5527.

Shrestha, H. K., Hwu, K. K., Wang, S. J., Liu, L. F. and Chang, M. C. (2008). Simultaneous detection of eight genetically modified maize lines using a combination of event- and construct-specific multiplex-PCR technique. *Journal of Agricultural and Food Chemistry*, 56, 19, 8962-8968.

Schweitzer, B. and Kingsmore, S. (2001). Combining nucleic acid amplification and detection. *Current Opinion in Biotechnology*, 12, 1, 21-27.

Stave, J. W. (1999). Detection of new or modified proteins in novel foods derived from GMO – future needs, *Food Control*, 10, 367-374.

Su, W., Song S., Long, M. and Liu, G. (2003). Multiplex polymerase chain reaction/membrane hybridization assay for detection of genetically modified

organisms. *Journal of Biotechnology*, 105, 227-233.

The Food and Agricultural Materials Inspection Center, Japan. (2002). Japanese Agricultural Standard (JAS) analytical test handbook: genetically modified food quality, labeling analysis manual for individual products. http://www.famic.go.jp/technical_information/jashandbook/index.html.

Torney, F., Moeller, L., Scarpa, A. and Wang, K. (2007). Genetic engineering approaches to improve bioethanol production from maize. *Current Opinion in Biotechnology*, 18, 193-199.

Vaïtilingom, M., Pijnenburg, H., Gendre, F. and Brignon, P. (1999). Real-Time Quantitative PCR Detection of Genetically Modified Maximizer Maize and Roundup Ready Soybean in Some Representative Foods. *Journal of Agricultural and Food Chemistry*, 47, 5261-5266.

Voller, A., Bartlett, A. and Bidwell, D. E. (1978). Enzyme immunoassays with special reference to ELISA techniques. *Journal of Clinical Pathology*, 31, 507-520.

Waiblinger, H. U., Ernst, B., Anderson, A. and Pietsch, K. (2008). Validation and collaborative study of a P35S and T-nos duplex real-time PCR screening method to detect genetically modified organisms in food products. *European Food Research and Technology*, 226, 1221–1228.

Watanabe, T., Kuribara, H., Mishima, T., Kikuchi, H., Kodama, T., Futo, S., Kasama, K., Toyota, A., Nouno, M., Saita, A., Takahashi, K., Hino, A., Akiyama, H., Maitani, T. and Kubo, M. (2004). New qualitative detection methods of genetically modified potatoes. *Biological & pharmaceutical bulletin*, 27, 9, 1333-1339.

Watson, J. D. and Click, F. H. C. (1953). Molecular structure of nucleic acids: a structure for deoxyribose nucleic acid. *Nature*, 171, 737-738.

Watson, J. D. and Click, F. H. C. (1953). Genetical implications of the structure of

deoxyribonucleic acid. *Nature*, 171, 964-967.

Weighardt, F., Barbati, C., Paoletti, C., Querci, M., Kay, S., De Beuckeleer, M. and Van den Eede, G. (2004). Real-time polymerase chain reaction-based approach for quantification of the pat gene in the T25 Zea mays event. *Journal of AOAC International*, 87, 6, 1342-1355.

Weng, H., Yang, L., Liu, Z., Ding, J., Pan, A. and Zhang, D. (2005). Novel reference gene, *high-mobility-group protein I/Y*, used in qualitative and real-time quantitative polymerase chain reaction detection of transgenic rapeseed cultivars. *Journal of AOAC International*, 88, 2, 577-584.

Wiedmann, M., Czajka, J., Barany, F. and Batt, C. A. (1992). Discrimination of *Listeria monocytogenes* from other *Listeria* species by ligase chain reaction. *Applied and Environmental Microbiology*, 58, 11, 3443-3447.

Wiedmann, M., Wilson, W. J., Czajka, J., Luo, J., Barany, F. and Batt, C. A. (1994). Ligase chain reaction (LCR)-overview and applications. *PCR Methods and Applications*, 3, 4, S51-64.

Windels, P., Bertrand, S., Depicker, A., Moens, W., Bockstaele, E. and Loose, M. (2003). Qualitative and event-specific PCR realtime detection methods for StarLink maize. *European Food Research and Technology*, 216, 259-263.

Wu, Y., Wu, G., Xiao, L. and Lu, C. (2007). Event-specific qualitative and quantitative PCR detection methods for transgenic rapeseed hybrids MS1xRF1 and MS1xRF2. *Journal of Agricultural and Food Chemistry*, 55, 21, 8380-8389.

Xu, X., Li, Y., Zhao, H., Wen S. Y., Wang, S. Q., Huang, J., Huang K. and Luo, Y. B. (2005). Rapid and reliable detection and identification of GM events using multiplex PCR coupled with oligonucleotide microarray. *Journal of Agricultural and Food Chemistry*, 53, 3789-3794

Xu, J., Miao, H., Wu, H., Huang, W., Tang, R., Qui, M., Wen, J., Zhu, S. and Li, Y. (2006). Screening genetically modified organisms using multiplex-PCR coupled with oligonucleotide microarray. *Biosensors & Bioelectronics*, 22, 71-77.

Xu, J., Zhu, S., Miao, H., Huang, W., Qiu, M., Huang, Y., Fu, X. and Li, Y. (2007). Event-specific detection of seven genetically modified soybean and maizes using multiplex-PCR coupled with oligonucleotide microarray. *Journal of Agricultural and Food Chemistry*, 55, 14, 5575-5579.

Yamaguchi, A., Shimizu, K., Mishima, T., Aoki, N., Hattori, H., Sato, H., Ueda, N., Watanabe, T., Hino, A., Akiyama, H. and Maitani, T. (2006). Detection method for genetically modified papaya using duplex PCR. *Shokuhin Eiseigaku Zasshi*, 47, 4, 146-150.

Yang, L., Xu, S., Pan, A., Yin, C., Zhang, K., Wang, Z., Zhou, Z. and Zhang, D. (2005a). Event specific qualitative and quantitative polymerase chain reaction detection of genetically modified MON863 maize based on the 5'-transgene integration sequence. *Journal of Agricultural and Food Chemistry*, 53, 24, 9312-9328.

Yang, L., Pan, A., Zhang, K., Yin, C., Qian, B., Chen, J., Huang, C. and Zhang, D. (2005b). Qualitative and quantitative PCR methods for event-specific detection of genetically modified cotton Mon1445 and Mon531. *Transgenic Research*, 14, 6, 817-831.

Yang, L., Chen, J., Huang, C., Liu, Y., Jia, S., Pan, L. and Zhang, D. (2005c). Validation of a cotton-specific gene, Sad1, used as an endogenous reference gene in qualitative and real-time quantitative PCR detection of transgenic cottons. *Plant Cell Reports*, 24, 4, 237-245.

Yang, L., Pan, A., Jia, J., Ding, J., Chen, J., Cheng, H., Zhang, C. and Zhang, D. (2005d). Validation of a tomato-specific gene, LAT52, used as an endogenous reference gene

in qualitative and real-time quantitative PCR detection of transgenic tomatoes. *Journal of Agricultural and Food Chemistry*, 53, 2, 183-190.

Yang, L., Pan, A., Zhang, H., Guo, J., Yin, C. and Zhang, D. (2006). Event-specific qualitative and quantitative polymerase chain reaction analysis for genetically modified canola T45. *Journal of Agricultural and Food Chemistry*, 54, 26, 9735-9740.

Yang, L., Guo, J., Pan, A., Zhang, H., Zhang, K., Wang, Z. and Zhang, D. (2007a). Event-specific quantitative detection of nine genetically modified maizes using one novel standard reference molecule. *Journal of Agricultural and Food Chemistry*. 55, 1, 15-24.

Yang, R., Xu, W., Luo, Y., Guo, F., Lu, Y. and Huang, K. (2007b). Event-specific qualitative and quantitative PCR detection of roundup ready event GT73 based on the 3'-integration junction. *Plant Cell Reports*, 26, 10, 1821-1831.

Yang, L., Guo, J., Zhang, H., Liu, J. and Zhang, D. (2008). Qualitative and quantitative event-specific PCR detection methods for oxy-235 canola based on the 3' integration flanking sequence. *Journal of Agricultural and Food Chemistry*. 56, 6, 1804-1809.

Yoshimura, T., Kuribara, H., Matsuoka, T., Kodama, T., Iida, M., Watanabe, T., Akiyama, H., Maitani, T., Furui, S. and Hino, A. (2005). Applicability of the quantification of genetically modified organisms to foods processed from maize and soy. *Journal of Agricultural and Food Chemistry*, 53, 6, 2052-2059.

LIST OF PUBLICATIONS

Mano, J., Shigemitsu, N., Futo, S., Akiyama, H., Teshima, R., Hino, A., Furui, S. and Kitta, K. (2009). Real-Time PCR Array as a universal platform for the detection of genetically modified crops and its application in identifying unapproved genetically modified crops in Japan. *Journal of Agricultural and Food Chemistry*, 57, 1, 26-37.

Mano, J., Oguchi, T., Akiyama, H., Teshima, R., Hino, A., Furui, S. and Kitta, K. (2009). Simultaneous Detection of Recombinant DNA Segments Introduced into Genetically Modified Crops with Multiplex Ligase Chain Reaction Coupled with Multiplex Polymerase Chain Reaction, *Journal of Agricultural and Food Chemistry*, 57, 7, 2640-2646.

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