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Short communication

Fatty acid content in muscles of amago salmon homozygous or heterozygous for a growth hormone transgene

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ABSTRACT

Fatty acids are a vital energy source in fish and are of significant importance to their physiological wellbeing. Amago salmon (*Oncorhynchus masou ishikawae*) transgenic for a growth hormone (GH) show both accelerated growth and altered fatty acid composition and content in liver tissues. In particular, they show a decrease in saturated fatty acids and monounsaturated fatty acids, and an increase in polyunsaturated fatty acids except for docosahexaenoic acid (22:6n−3) and eicosapentaenoic acid (20:5n−3). Furthermore, transgenic fish have decreased levels of serum glucose, triacylglycerol and an increase in 3-hydroxybutyric acid, generally considered a starvation marker. As liver tissue is physiologically connected to muscle tissue, here we examined the effects of GH transgenesis on fatty acid contents in muscles of homozygous and heterozygous GH transgenic fish. The major monounsaturated fatty acids oleic acid (18:1n−9) and palmitoleic acid (16:1n−7) were slightly higher in the control, whereas polyunsaturated fatty acids, except 22:6n−3 and 20:5n−3, were significantly greater in the transgenic fish ($P < 0.05$), similar to the results from the liver. However, by contrast to the liver, the major saturated fatty acids palmitic acid (16:0) and stearic acid (18:0) and polyunsaturated fatty acids (22:6n−3) and (20:5n−3) were significantly higher ($P < 0.05$) in the transgenic fish than in the controls.

1. Introduction

GH transgenesis in fish almost invariably causes increased growth performance, for example, GH transgenic salmon typically show a 6 to 11 fold increase in body weight and occasionally a 40 fold or more increase has been reported (Devlin et al., 1994; Rahman et al., 1998). We have also generated fast-growing GH transgenic amago salmon, and showed that the fish had down-regulation of Δ-6 fatty acyl desaturase (Δ6FAD) expression using functional microarray analysis (Mori et al., 2007). This enzyme is important for the modification of polyunsaturated fatty acids (PUFAs) in many vertebrates (Zheng et al., 2004), and a decrease in its expression causes changes to the levels of various PUFAs. In a subsequent study of the effect of GH transgenesis on metabolic processes, we produced homozygous (Tg/Tg) and heterozygous (Tg/+) GH transgenic amago salmon (Kurata et al., 2012). Analysis of these fish showed that serum IGF-I concentrations were significantly higher in the transgenic fish than in the controls. The highest serum GH1 concentrations occurred in the Tg homozygotes, with a significantly lower level in heterozygotes and the lowest level in controls. Moreover, an iTRAQ-MS/MS proteome and microarray analysis showed that these GH transgenic amago salmon had a drastic decrease in the amount of fat tissue that accumulated around the pyloric caeca compared to the controls, and also had down-regulation of fatty acid synthase (FAS) in the pituitary (Kurata et al., 2012).

We examined metabolic processes in the liver tissue of GH transgenic amago salmon and found an enhanced catabolic reaction of fatty acids compared to controls. This change in catabolism caused an increase in β-oxidation of saturated (SFAs) and monounsaturated fatty acids (MUFAs) in homozygous (Tg/Tg) and heterozygous (Tg/+) amago salmon compared to the controls (Sugiyama et al., 2012). Expression of the Mid1 interacting protein 1 gene (*Mid1ip1*), which is important in enhancing de novo fatty acid synthesis, was down-regulated, and an increase in 3-hydroxybutyric acid (a ketone body) was observed in the livers of the GH transgenic fish. These results indicate that the liver tissue from GH transgenic fish is in a state of starvation. The amounts of SFAs and MUFAs in the livers were found to decrease in the order homozygous (Tg/Tg) and heterozygous (Tg/+) GH transgenic, and control fish. By contrast, the amounts of n−3 PUFA rose in this order.

Fish lipids are rich in PUFAs, and these have important roles in regulation of inflammation (Arts and Kohler, 2009) and the immune system (Rowley et al., 1995). Therefore, analysis of fatty acid composition in lipids is a valuable means of understanding physiological changes and the health condition of both mammals and fish. Therefore, analysis of the effect of GH transgenesis on lipid metabolism in muscle tissue will

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provide valuable insights into the health and physiological condition of the fish.

2. Material and methods

2.1. Experimental animals

GH transgenic amago salmon were generated by injecting *OnMTGH1* gene construct into fertilized eggs (Devlin et al., 1994). In this experiment, we used heterozygous (Tg/+) GH transgenic amago which were produced by fertilizing domestic-type sperms with eggs collected from transgenic fish containing the *OnMTGH1*. Homozygous (Tg/Tg) GH transgenic fish were produced by mixing eggs and sperm obtained from heterozygous (Tg/+) fish. The fish were reared in equal densities in circulating tanks under a natural light cycle, and fed to satiation with a stage-specific commercial diet for juvenile fish (1-4CDX and Masu 5-8p from Nippon Formula Feed Mfg. Co., Ltd) until the end of the experiment (about 6 months). The mean weights of the homozygous (Tg/Tg), heterozygous (Tg/+) (note that all the heterozygotes were produced using eggs from transgenic fish and sperm from wild type), and age control (+/+) fish used in this experiment were 131 g, 109 g, and 85 g, respectively. Details of the production and detection of the transgenic fish using PCR were described in a previous study (Sugiyama et al., 2012).

Muscle tissues were obtained from 5–6 specimens of homozygous (Tg/Tg), heterozygous (Tg/+), and control (+/+) amago, frozen in liquid nitrogen, and stored at −80 °C until analysis. Extraction and purification of total lipids were performed following the method of Folch et al.

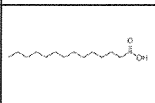
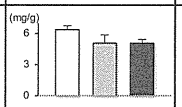
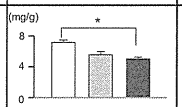
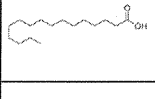
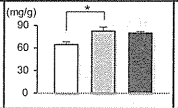
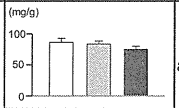
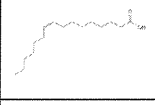
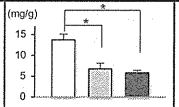
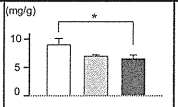
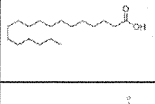
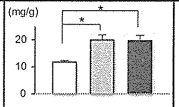
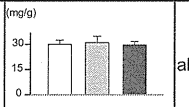
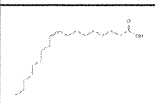
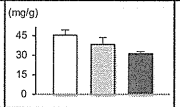
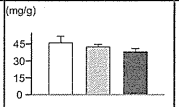
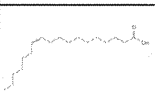
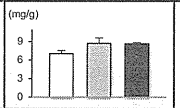
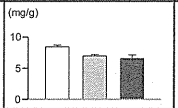
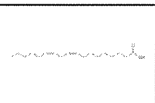
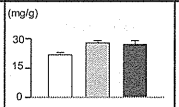
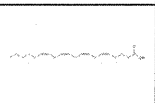
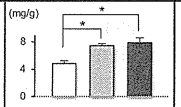
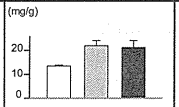
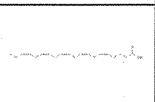
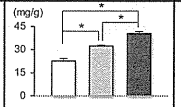
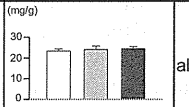
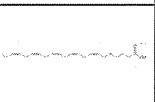
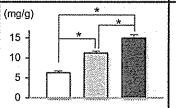
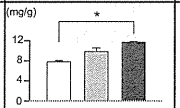
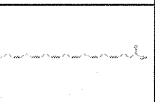
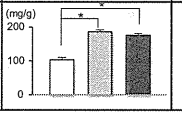
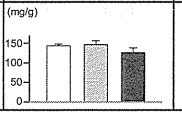
Tissue		Muscle		Liver	
Common name Numerical symbol (Content of diet)	Structure	Content (mg/g)	Pattern	Content (mg/g)	Pattern
Myristic acid 14:0 (2.1 %)			Down		Down
Palmitic acid 16:0 (21.3 %)			Up		No alteration
Palmitoleic acid 16:1n-7 (3.4 %)			Down		Down
Stearic acid 18:0 (5.6 %)			Up		No alteration
Oleic acid 18:1n-9 (17.4 %)			Down		Down
cis-Vaccenic acid 18:1n-7			Up		Down
Linoleic acid 18:2n-6 (24.3 %)			Up		Up
Arachidonic acid 20:4n-6 (1.0 %)			Up		Up
Eicosapentaenoic acid 20:5n-3 (4.2 %)			Up		No alteration
Docosapentaenoic acid 22:5n-3 (1.1 %)			Up		Up
Docosahexaenoic acid 22:6n-3 (10.8 %)			Up		Down

Fig. 1. Fatty acid contents (mg/g) in muscle tissue from homozygous (black), heterozygous (gray) of GH transgenic amago salmon, and from controls (white), compared with those in liver tissue (Sugiyama et al., 2012). Contents of fatty acids were calculated using heptadecanoic acid (17:0) as an internal standard. The changes in content are shown as Up or Down in comparison to the control. Data are presented as means ± standard error. Asterisk "*" indicates a significant difference ($P < 0.05$). Figure in parentheses shows fatty acid content (%: W/W) of the fish diet.

(1957). Fatty acid methyl esters were prepared by transesterification with 15% boron trifluoride in methanol. Fatty acid composition was analyzed by detection on a 5890 series II GC (Agilent Technologies, Inc., Santa Clarita, CA, U.S.A.) equipped with a flexible fused-silica (FFS) capillary column (ULBON-HR-SS-10; 0.25 mm I.D. ×50 m, Shinwa Chemical Industries, Inc., Tokyo, Japan). Details of the procedure were described in a previous study (Sugiyama et al., 2012).

2.2. Statistical analysis

Values for fatty acid contents (mg/g) were expressed as means ± SE, and data were analyzed by one-way analysis of variance followed by a posteriori comparison of the significant ANOVA results using Bonferroni or Dunnett's T3. Levene's multiple comparison test for variances indicated that the assumption of homogeneity of variances was rejected at the 5% significance level. For our statistical decisions, we used Dunnett's T3 multiple comparison test for means which is robust for testing means under heterogeneous variance. Significance was defined as $P < 0.05$.

3. Results and discussion

In our previous study, we found that the levels of serum triacylglycerol (TAG) and glucose in GH transgenic amago salmon were significantly lower than those of control fish (+/+). Further, the expression levels of the glucose starvation response genes, glucose regulated protein 78 kDa (GRP78) was over 10 folds higher than those of the control liver. Moreover, genes involved in fatty acid catabolism were up-regulated in the transgenic fish including long-chain-fatty-acid–CoA ligase 1 (*ACSL1*) (934 folds higher than control) and acyl-coenzyme A oxidase 3 (*ACOX3*) (14.3 folds); the ketone body compound 3-hydroxybutyric acid is present at higher levels in the liver of GH transgenic amago salmon than control fish (+/+). However, expression of *Mid1ip1* is down-regulated (107.5 folds) in transgenic salmon (Sugiyama et al., 2012). *Mid1ip1* is an important factor in the activation of acetyl-CoA carboxylase (ACC), which is known to act as a rate-limiting enzyme for de novo fatty acid synthesis (Kim et al., 2010). This may indicate that lipid storage in the liver of GH transgenic fish is lower than in control fish (+/+). The adipose tissue of GH transgenic fish is somewhat smaller than in control fish (+/+) (Kurata et al., 2012). Overall, therefore, the available information from studies on the liver of transgenic fish suggests that they have a reduced level of fatty acids as well as of glucose, presumably as a consequence of changes in glucose and lipid metabolism due to GH overexpression (Sugiyama et al., 2012). In light of the evidence of changes in the liver, we were interested in determining whether the muscle tissue of GH transgenic amago salmon also displayed the physiological changes associated with starvation.

Eleven major fatty acids were detected by gas chromatographic analysis of the total lipid extracts from the muscles of homozygous (Tg/Tg), heterozygous (Tg/+) and control fish (+/+). The relative fatty acid contents (mg/g) of the muscles in the homozygous (Tg/Tg) and heterozygous (Tg/+) fish are compared to controls in Fig. 1, and those of liver data were also quoted from our previous report (Sugiyama et al., 2012). The muscle tissue used in this experiment was obtained from the fish used for fatty acid analysis of liver tissue in a previous report (Sugiyama et al., 2012).

In some comparisons, there were clear, statistically significant differences among the groups; in others, the differences among homozygous (Tg/Tg), heterozygous (Tg/+) and control fish (+/+) were not significant, however, there was a consistent trend in the pattern of change between the genotypes.

Thus, for two of the MUFAs identified here, there was a trend to higher levels in control fish (+/+) than in the transgenic fish: palmitoleic acid (16:1n−7) and oleic acid (18:1n−9) both occurred in greater amounts in muscle and liver tissue from control fish (+/+) than from transgenic fish, and significantly so in the case of 16:1n−7 ($P < 0.05$). However, a third MUFA, cis-vaccenic acid (cis-18:1n−7), which is the last substrate in the fatty acid (n−7 family) synthetic pathway (Fig. 2), showed the opposite trend and increased in the muscle from transgenic fish (Fig. 1). Of the three MUFAs, 18:1n−9 was present in the largest amounts, followed by 16:1n−7, and finally cis-18:1n−7 (Fig. 1). We found decreased muscle content for two MUFAs (16:1n−7 and 18:1n−9) but not of 18:1n−7, and increased content of 5 PUFAs in the muscle (Fig. 1). These changes are similar to those reported in rainbow trout subjected to starvation (Johansson and Kiessling, 1991). Cis-18:1n−7 was also associated with the production of high concentrations of adiponectin (Takkunen et al., 2014). Adiponectin in skeletal muscle activates AMP-activated protein kinase, thereby directly regulating glucose metabolism and insulin sensitivity in vitro and in vivo (Yamauchi et al., 2002). These results are consistent with adaptation to the low glucose concentration under starvation in the muscle tissue from transgenic fish.

Three SFAs were identified here. Two of these, palmitic acid (16:0) and stearic acid (18:0), showed a significant increase in the muscle from transgenic fish ($P < 0.05$). The third, myristic acid (14:0) showed the opposite trend and fell consistently from control (+/+) to homozygous fish (Tg/Tg).

Five PUFAs were identified in this analysis and all showed a trend toward increasing content in the muscles of transgenic fish. For four of these fatty acids, arachidonic acid (20:4n−6), eicosapentaenoic acid (20:5n−3), docosapentaenoic acid (22:5n−3), and docosahexaenoic acid (22:6n−3), the increases were significant ($P < 0.05$) in the muscle from transgenic fish. Although linoleic acid showed the same trend as other PUFAs, there were no significant inter-genotype differences.

Although the muscle contents of major SFAs (16:0 and 18:0) and PUFAs (22:6n−3 and 20:5n−3) were increased in the GH transgenic fish (Figs. 1 and 2), only 18:2n−6 of the identified PUFAs did not show a significant increase in muscle tissue (Fig. 1), but rather was found at similar levels in all three genotypes examined. This compound comprises 24% (w/w) of the total fatty acids in the food as shown in Fig. 1 (Sugiyama et al., 2012). With respect to PUFA content in the muscle tissue from GH transgenic fish, similar fatty acid content patterns were seen as in liver tissue except for 22:6n−3 and 20:5n−3 (Fig. 1) (Sugiyama et al., 2012). Interestingly, 22:6n−3 showed the opposite trend between muscle and liver tissues. However, 22:6n−3 in the food comprises over 10% (w/w), this might indicate that content of 22:6n−3 in the transgenic fish having a good appetite is greater than in the control fish (+/+). Meanwhile, although we found liver from GH transgenic amago showing vasodilation and presumably angiogenesis (Sugiyama

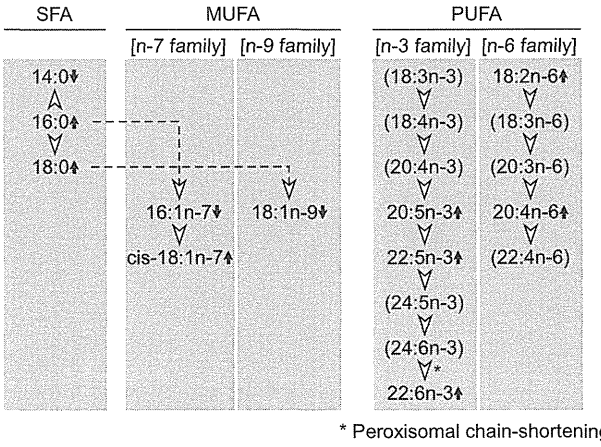


Fig. 2. Flow chart depicting the metabolic pathways of fatty acids in the muscle tissue of GH transgenic amago salmon. Up and down arrows beside fatty acids indicate increased and decreased contents of fatty acids in the muscle of GH transgenic amago salmon, respectively. Asterisk "*" indicates the process of peroxisomal chain shortening.

et al., 2010), there is no notable morphological abnormality in the muscle from transgenic fish. Further, there is a report that 22:6n−3 was significantly inversely-correlated with circulating inflammatory protein (CRP) (Takkunen et al., 2014). This may lead the decrease of 22:6n−3 content in the liver tissue from GH transgenic fish.

In this analysis of fatty acid contents in the tissues, it is predicted that GH transgenic fish have the potential to adapt to starvation in the muscle through cis-18:1n−7. Decrease of 22:6n−3 in the liver tissue from the transgenic fish may also indicate inflammatory reaction.

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ORIGINAL PAPER

A novel trait-specific real-time PCR method enables quantification of genetically modified (GM) maize content in ground grain samples containing stacked GM maize

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Abstract Stacked genetically modified (GM) maize is increasingly produced; thereby, current event-specific quantitative real-time polymerase chain reaction (qPCR) methods have led to the overestimation of GM organism (GMO) content compared with the actual weight/weight percentage of GM organism in maize samples. We developed a feasible qPCR method in which the GMO content is calculated based on the quantification of two herbicide-tolerant trait genes, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (*cp4epsps*) and phosphinothricin *N*-acetyl-transferase from *Streptomyces viridochromogenes* (*pat*) to quantify the GMO content in ground grain samples containing stacked GM maize. The GMO contents of two genes were quantified using a plasmid calibrant and summed for quantification of total GMO content. The trait-specific method revealed lower biases for examination of test samples containing stacked GM maize compared with the event-specific method. Our results clearly show that the trait-specific method is not only simple and cost-effective, but also useful in quantifying the GMO content in ground grain samples containing stacked GM maize, which are expected to be major events in the near future. The developed method would be the only feasible way to conduct the quantification of GMO content in the ground maize samples containing stacked GM maize for the verification of the labeling regulation.

Keywords Genetically modified maize · qPCR · Trait-specific method · Stacked GM maize

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Introduction

In recent years, an increasing number of genetically modified (GM) crops have been developed using recombinant DNA technology and are widely cultivated as sources of food and feed in many countries. GM crops have generally been assessed and authorized for use as food by administrative authorities. However, the use of GM crops for food remains controversial among consumers in many countries. Labeling of GM foods allows consumers to make informed food choices. Therefore, many countries have mandated the labeling of foods containing a specified threshold level of GM crops (0.9 % in the European Union, 3 % in Korea and 5 % in Japan) [1]. To monitor the content of GM crops such as maize [2–8], soybean [2–4, 8–10] and other crops [11–13] in foods, in general, the quantitative real-time polymerase chain reaction (qPCR) has been used. In several

countries including Japan, the regulatory threshold levels of GMO content are evaluated on a weight/weight (w/w) basis and are calculated based on the GM event-specific DNAs to taxon-specific DNA ratio measured using qPCR.

Recently, the production of stacked GM maize grains, with two or more GM events for enhanced production efficiency [14], has been increasing worldwide. The GMO content of maize samples containing stacked GM maize is generally overestimated when determined using qPCR methods, as compared to the actual w/w percentage of GM maize, because a kernel of stacked GM maize contains the GM-specific DNAs in proportion to the number of GM events. To avoid overestimation, we developed an individual kernel detection method that involves multiplex real-time PCR using the extracted DNA from individual ground maize kernels [15–18]. This detection system has already been implemented in Japan as an official GM maize detection method [19]. Moreover, a GMO content evaluation method based on group testing strategy [20–22] was recently developed [23]. In this method, GMO content is statistically evaluated based on qualitative PCR for multiple small portions, consisting of 20 maize kernels. However, these methods are not applicable to ground grain samples such as corn grits, corn flour and corn meal. Moreover, both methods are time consuming and require additional equipment with large sample numbers. A simpler, time-saving and cost-effective method is required for roughly quantifying GMO content in maize samples containing stacked GM maize.

We previously determined the GM maize content on a kernel basis and the events of GM maize kernels in non-identity-preserved (IP) maize samples imported from the USA in 2005 and 2009 using an individual kernel detection system [24, 25]. The main GM maize events detected in the non-IP maize samples in 2009 were MON88017, MON810 × MON88017, NK603, MON810, TC1507 × DAS59122, MON810 × NK603, TC1507, DAS59122 and MON863. With the exception of single GM maize events of MON810 and MON863, these GM maize events contain a herbicide-tolerant trait gene encoding either 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (*cp4epsps*) or phosphinothricin *N*-acetyl-transferase from *Streptomyces viridochromogenes* (*pat*) (Supplementary Table S1). Moreover, many other stacked GM maize events detected in the non-IP maize samples in 2009 also contain *cp4epsps* or *pat*. Thus, we hypothesized that the GMO content in ground maize samples containing stacked GM maize might be quantified from the sum of *pat* and *cp4epsps* contents. This hypothetical method, termed the trait-specific method, might be applicable to ground samples, whereas the individual kernel detection method and the group testing method are applicable only to kernel samples. Moreover, the hypothetical method for the determination of trait-specific gene might be also applicable to kernel samples, with the advantages of easy sample preparation and enhanced cost-effectiveness. To date, *pat* or *cp4epsps* is used in most GM lines as an herbicide-tolerant trait gene. In this study, we developed a trait-specific method that can quantitate GMO content by measuring only *cp4epsps* and *pat* using qPCR and demonstrated that the developed method is appropriate for approximate quantification of GMO content in ground grain samples containing stacked GM maize by evaluating the performance of the developed method by quantitating five test samples in comparison to the event-specific method.

Materials and methods

Maize materials

The MON88017, MON810, MON863, NK603, MON 88017 × MON810, MON810 × NK603 and non-GM maize seeds were kindly provided by Monsanto Co. (St. Louis, MO). Seeds of TC1507 and DAS59122 were kindly provided by Pioneer Hi-Bred International (Johnston, IA). The 5 % MON810 certified reference material (CRM), 5 % NK603 CRM and 10 % TC1507 CRM were purchased from Sigma-Aldrich (St. Louis, MO).

DNA extraction

The maize seeds were ground using a Mixer Mill MM200 (Retsch, Haan, Germany). Genomic DNA was extracted and purified from 1 g of ground maize powder using a DNeasy Plant Maxi Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, with the following modifications. Five milliliters of AP1 buffer (QIAGEN) and 10 μL of 100 mg/mL RNase (QIAGEN) were added to the sample and vortexed thoroughly and then incubated at 65 °C for 1 h. The mixture was incubated at 65 °C for another 1 h after the addition of 200 μL of Proteinase K (QIAGEN). During incubation, the mixture was mixed several times by vortexing the tubes. After incubation, 1.8 mL of AP2 buffer (QIAGEN) was added to the mixture and vortexed and then incubated on ice for 15 min. The mixture was centrifuged at 2,300×*g* for 15 min at room temperature in a swing-out rotor, and the supernatant was applied to a QIAshredder Maxi spin column. The column was centrifuged at 2,300×*g* for 5 min at room temperature, and 5.1 mL of AP3/E buffer (QIAGEN) was added to 3.4 mL of flow-through solution, followed by vortexing thoroughly. The mixture was applied to a DNeasy Maxi spin column, followed by centrifugation at 2,300×*g* for 5 min at room temperature. The column was washed with 12 mL of AW buffer (QIAGEN) and then centrifuged at 2,300×*g* for 15 min at room temperature. To elute the DNA, 1 mL of pre-warmed distilled water (65 °C)

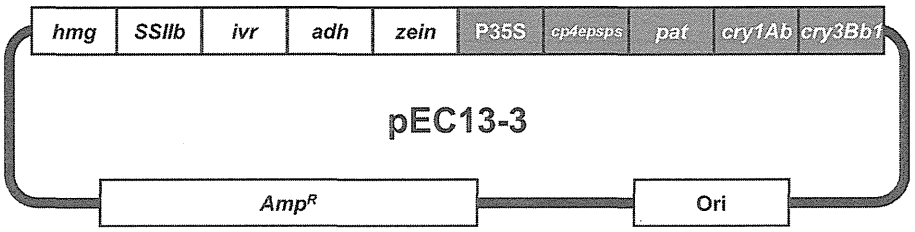

Fig. 1 Schematic diagram of pEC13-3 integrating ten fragments (*hmg*, *SSIIb*, *ivr*, *adh*, *zein*, P35S, *cp4epsps*, *pat*, *cry1Ab* and *cry3Bb1*). Amp^R, ampicillin resistance gene. Ori, origin of replication

was added to the column. After incubation at room temperature for 5 min, the column was centrifuged at 2,300×*g* for 10 min at room temperature. An equal amount of isopropyl alcohol was added to the eluted solution, and the mixture was mixed thoroughly. After incubation at room temperature for 5 min, the mixture was centrifuged at 12,000×*g* at 4 °C for 15 min. The pellet was rinsed with 500 μL of 70 % (v/v) ethanol and centrifuged at 12,000×*g* at 4 °C for 3 min. The supernatant was discarded and the precipitate was dried. The DNA was dissolved in 100 μL of distilled water.

DNA concentrations were determined by measuring UV absorption at 260 nm with a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Samples were diluted to 20 ng/μL with sterile distilled water. The extracts (600 ng) were analyzed by electrophoresis on a 1.0 % agarose gel containing Midori Green Advanced DNA strain (NIPPON Genetics, Tokyo, Japan).

Preparation of plasmid DNA

To quantitate the GM maize content, we prepared plasmid DNA as a calibrant. Ten targeted DNA fragments consisting of five reference genes [high mobility group protein (*hmg*, AJ131373), starch synthase IIb (*SSIIb*, NM_001111410), invertase A (*ivr*, ZMU16123), alcohol dehydrogenase 1 (*adh*, X04050) and delta zein protein (*zein*, FJ557103)] and five GM genes [cauliflower mosaic virus 35S promoter (P35S, AB863197), *cp4epsps* (AY125353), *pat* (DQ156557) and two Bt toxins (*cry1Ab*; AY326434 and *cry3Bb1* [26])] were incorporated (as shown in Fig. 1) by PCR as described previously [2] using appropriate primers. The resultant fragment was ligated into pUC19, and its sequence was confirmed by nucleotide sequence analyses and designated as pEC13-3. The cells of *Escherichia coli* DH5α were transformed using pEC13-3. The plasmid was extracted with a Plasmid Mega Kit (QIAGEN) and purified by ultracentrifugation with cesium chloride. The purified pEC13-3 was cut by *Nde*I, and the resultant linearized plasmid DNA was purified again by ultracentrifugation with cesium chloride. The copy number of purified pEC13-3 was estimated as that of *SSIIb* by qPCR as described previously [2], and plasmid DNA was diluted with 5 ng/μL ColE1 plasmid solution in tris-ethylenediaminetetraacetic acid buffer (Nippon Gene, Tokyo, Japan) to 20, 125, 1,500, 20,000 and 250,000 copies per 2.5 μL.

Table 1 The GM maize event and content (%) of test samples prepared by mixing the ground samples of several GM maize and non-GM maize

GM maize event	Sample number				
	1	2	3	4	5
MON88017	2.0	1.3	1.3	1.5	–
MON810 × MON88017	–	1.2	1.3	1.5	3.0
NK603	1.0	0.6	0.7	–	–
MON810	0.9	0.5	0.7	–	–
TC1507 × DAS59122	–	0.4	0.5	1.0	1.0
MON810 × NK603	–	0.4	0.5	1.0	1.0
TC1507	0.6	0.3	–	–	–
DAS59122	0.3	0.2	–	–	–
MON863	0.2	0.1	–	–	–
Total	5.0	5.0	5.0	5.0	5.0

Preparation of test samples

To prepare the five test samples, the ground grain samples of several GM maize events were mixed with ground non-GM maize at 5 % (w/w) GMO content (Table 1). Genomic DNA was extracted from each test sample in three parallels and diluted to 20 ng/μL with sterile distilled water.

qPCR assay

qPCR assay was performed by trait- and event-specific qPCR methods using an ABI PRISM™ 7900HT Sequence Detection System (Life Technologies, Carlsbad, CA). For the trait-specific method, five sets of primer pairs and probe (*hmg* [27–30], *SSIIb* [31], *ivr* [32], *adh* [32] and *zein* [32]) were identical with those in previous reports. Two sets of primer pairs and probe (*cp4epsps* and *pat*) were designed using Primer Express software (Life Technologies) (Table 2). These sets of primer pairs and probe were evaluated for PCR efficiency and linearity of calibration curves for each gene in pEC13-3. Trait-specific quantification was performed by quantitating *cp4epsps* and *pat* in each DNA extracted in three parallels. A 25 μL volume of the reaction mixture contained 2.5 μL of template DNA, 12.5 μL of TaqMan® Universal PCR Master Mix (Life Technologies), 0.5 μM of each primer and

Table 2 Primers and probes used for qPCR assay

Target gene	Accession number	Primer or probe name	Primer or probe sequence (5′–3′)	Amplicon size (bp)
reference gene				
hmg	AJ131373	hmg-F	TTGGACTAGAAATCTCGTGCTGA	79
		hmg-R	GCTACATAGGGAGCCTTGTCCT	
		hmg-P	FAM-CAATCCACACAAACGCACGCGTA-TAMRA	
SSIIb	NM_001111410	SSIIb3-5′	CCAATCCTTTGACATCTGCTCC	114
		SSIIb3-3′	GATCAGCTTTGGGTCCGGA	
		SSIIb-P	FAM-AGCAAAGTCAGAGCGCTGCAATGCA-TAMRA	
ivr	ZMU16123	ivr-F	CGCTCTGTACAAGCGTGC	135
		ivr-R	GCAAAGTGTTGTGCTTGGACC	
		ivr-P	FAM-CACGTGAGAATTTCCGTCTACTCGAGCCT-TAMRA	
adh	X04050	adh-F	CGTCGTTTCCCATCTCTTCCTCC	103
		adh-R	CCACTCCGAGACCCTCAGTC	
		adh-P	FAM-AATCAGGGCTCATTTTCTCGCTCCTCA-TAMRA	
zein	FJ557103	zein-F	GCCATTGGGTACCATGAACC	104
		zein-R	AGGCCAACAGTTGCTGCAG	
		zein-P	FAM-AGCTTGATGGCGTGTCCGTCCCT-TAMRA	
trait gene				
cp4epsps	AY125353	cp4epsps-F	TTCACGGTGCAAGCAGCC	82
		cp4epsps-R	GACTTGTCGCCGGGAATG	
		cp4epsps-P	FAM-CGCAACCGCCCGCAAATCC-TAMRA	
pat	DQ156557	pat-F	GGCCTTCCAAACGATCCAT	96
		pat-R	CCATCCACCATGCTTGTATCC	
		pat-P	FAM-ATGAGGCTTTGGGATACACAGCCCG-TAMRA	

0.2 μM of probe. PCR conditions were as follows: 2 min at 50 °C, 95 °C for 10 min followed by 45 cycles of 30 s at 95 °C and 1 min at 59 °C. Standard curves were calibrated using the five concentrations of plasmid DNAs, such as 20, 125, 1,500, 20,000 and 250,000 copies per reaction. The no-template control containing 5 ng/μL ColE1 plasmid was also prepared as the negative control for analysis. For event-specific quantification, event-specific sequences of MON88017, MON810, MON863, NK603, TC1507 and DAS 59122 were quantitated in each DNA extracted in three parallels according to the methods reported by the European Commission's Joint Research Centre [27–30, 33, 34]. Standard curves were calibrated by using genomic DNA extracted from 5 % MON810 CRM, 5 % NK603 CRM and 10 % TC1507 CRM, and 10 % MON88017, 5 % DAS59122 and 10 % MON863 prepared from ground powders of GM maize and non-GM maize. Triplicate reactions for each DNA extracted in three parallels were conducted using trait- and event-specific qPCR.

Data analysis

For trait-specific quantification, the baseline was set to cycles 3 through 15 and the ΔRn threshold for plotting quantification cycle (C_q) values was set to 0.2 during exponential amplification. The PCR efficiency (E, %) of reference genes was calculated using the slope of the standard curve according to the following formula:

$$\text{PCR efficiency } (E, \%) = \left[10^{(-1/\text{slope})} - 1\right] \times 100 \quad (1)$$

The ratio of the copy number of *hmg* and trait gene (*cp4epsps* and *pat*) in GM maize seeds, defined as the conversion factor (C_f), was calculated using the following formula:

$$C_f = \frac{\text{copy number of trait gene in the DNA extracted from GM maize seeds}}{\text{copy number of } hmg \text{ in the DNA extracted from GM maize seeds}} \quad (2)$$

To prevent overestimation of GMO content, we used the corrected C_f value calculated using following formula:

$$\text{Corrected } C_f = \sum \left(C_{f(\text{trait})} \times x\right) \quad (3)$$

Table 3 The corrected C_f calculated from the C_f value and the rate of each GM maize event containing *cp4epsps* or *pat*

GM maize event	*cp4epsps*		*pat*	
	C_f	x[a]	C_f	x[a]
MON88017	0.30	0.38	–	–
MON810 × MON88017	0.28	0.34	–	–
NK603	0.72	0.18	–	–
MON810	–	–	–	–
TC1507 × DAS59122	–	–	0.61	0.42
MON810 × NK603	0.71	0.10	–	–
TC1507	–	–	0.30	0.38
DAS59122	–	–	0.29	0.20
MON863	–	–	–	–
Corrected C_f[b]	0.41		0.43	

[a] x is the rate of a GM maize event in all main GM maize events containing *cp4epsps* or *pat*, which was calculated using the data of GMO content in the non-IP maize sample imported from the USA in 2009. The sum of x of all main GM maize events containing *cp4epsps* or *pat* was 1.00

[b] The corrected C_f was calculated from the sum of the product of each C_f and x as follows: for *cp4epsps*, the corrected $C_{f(cp4epsps)} = C_{f(MON88017)}$ (0.30) × $x_{(MON88017)}$ (0.38) + $C_{f(MON810 \times MON88017)}$ (0.28) × $x_{(MON810 \times MON88017)}$ (0.34) + $C_{f(NK603)}$ (0.72) × $x_{(NK603)}$ (0.18) + $C_{f(MON810 \times NK603)}$ (0.71) × $x_{(MON810 \times NK603)}$ (0.10) = 0.41; for *pat*, the corrected $C_{f(pat)} = C_{f(TC1507 \times DAS59122)}$ (0.61) × $x_{(TC1507 \times DAS59122)}$ (0.42) + $C_{f(TC1507)}$ (0.30) × $x_{(TC1507)}$ (0.38) + $C_{f(DAS59122)}$ (0.29) × $x_{(DAS59122)}$ (0.20) = 0.43

where $C_{f(trait)}$ is each C_f value for *cp4epsps* or *pat* in a GM maize event containing *cp4epsps* or *pat* and x is the rate of a GM maize event in all main GM maize events containing *cp4epsps* or *pat*, which was calculated using the data of GMO content in the non-IP maize sample imported from the USA in 2009 [25] (Table 3). The corrected C_f was calculated by summing the product of $C_{f(trait)}$ and × ($C_{f(trait)}$ × x) for each GM maize event. The GMO content (%) of a sample for each trait gene was calculated using the following formula:

$$\text{GMO content (\%)} = \frac{\text{copy number of trait gene in the DNA extracted from a sample}}{\text{copy number of } hmg \text{ in the DNA extracted from a sample} \times \text{corrected } C_f} \times 100 \quad (4)$$

Total GMO content was calculated by summing the GMO content for *cp4epsps* and *pat*.

For event-specific quantification, the baseline was set to cycles 3 through 15 and the ΔRn threshold for plotting C_q values was set to 0.1–0.5 during exponential amplification. The GMO content (%) of a sample for each GM sequence was calculated using the following formula:

$$\text{GMO content (\%)} = \frac{\text{copy number of event-specific sequence in the DNA extracted from a sample}}{\text{copy number of endogenous gene in the DNA extracted from a sample}} \times 100 \quad (5)$$

Table 4 The PCR efficiencies (*E*) and linearity (R^2) of calibration curves for five reference genes and two herbicide-tolerant trait genes in pEC13-3

Target gene	*E*		R^2	
	Mean	RSD (%)	Mean	RSD (%)
Reference gene				
Hmg	100.7	2.2	0.9990	0.08
SSIIb	98.7	2.3	0.9980	0.10
Ivr	96.7	2.4	0.9960	0.39
Adh	96.7	3.2	0.9961	0.13
Zein	97.0	2.0	0.9989	0.06
Trait gene				
Cp4epsps	96.7	3.7	0.9992	0.05
Pat	94.7	3.0	0.9992	0.03

Total GMO content was calculated by summing the GMO content for each event-specific sequence.

Results and discussion

Construction of plasmid DNA

Plasmid pEC13-3 was constructed by tandem integration of ten PCR products amplified from five reference genes (*hmg*, *SSIIb*, *ivr*, *adh* and *zein*) and five GM genes (P35S, *cp4epsps*, *pat*, *cry1Ab* and *cry3Bb1*) (Fig. 1) as a calibrant. The purified pEC13-3 was diluted to 20, 125, 1,500, 20,000 and 250,000 copies per 2.5 μL, equivalent to 0.12, 0.72, 8.6, 115 and 1,440 ng of genomic DNA of F1 GM maize seed, respectively, based on the genome size of maize (the diploid DNA content per nucleus, 5.75 pg/2C) [35]. The dilution series was sufficient to quantitate GM maize content from 0.23 to 100 % in 50 ng of genomic DNA.

The PCR efficiencies of reference genes in pEC13-3 were calculated by the designated qPCR system (Table 4). Among the five reference genes, *hmg* showed the highest PCR efficiency [E = 100.7 %, relative standard deviation (RSD) = 2.2 %], followed by *SSIIb* (98.7 %), *zein* (97.0 %), *adh* (96.7 %) and *ivr* (96.7 %), with RSD ranging from 2.0 % to 3.4 %. Moreover, the standard curve of *hmg* showed greater linearity (R^2 = 0.9990, RSD = 0.08 %) than that of the other reference genes (0.9960–0.9989),

with RSD ranging from 0.06 to 0.39 %. The relative copy number of *hmg* in some GM maize events was compared to that of *SSIIb*, which is a standard reference gene of maize used in Japanese regulation (Supplementary Table S2), where that of *hmg* or *SSIIb* in the non-GM maize was 1.00. The resulting data of quadruplicate reactions showed that the mean of the relative copy numbers of *hmg* (1.21, RSD = 0.6–12.5 %) was lower than that of *SSIIb* (1.36, RSD = 4.1–9.6 %). The estimated copy number of *SSIIb* in GM maize was higher than non-GM maize as compared to that of *hmg*. These results suggest that *hmg* is the most appropriate reference gene for the designated qPCR system in this method. On the other hand, the PCR efficiencies of the two herbicide-tolerant trait genes, *cp4epsps* and *pat* (96.7 and 94.7 %, respectively), were comparable to the reference genes (96.7–100.7 %), and both standard curves of the two genes showed excellent linearity ($R^2 = 0.9992$) (Table 4).

Measurement of conversion factor

To determine the C_f value required for the trait-specific qPCR method, the copy number of *hmg* and the trait gene in the genomic DNA extracted from GM maize seed were each measured. All experiments were repeated three times, and the mean values were set as the C_f value (Table 3). The C_f value from whole seed [$C_{f(seed)}$] should be a mean value between the C_f value from embryo [$C_{f(emb)}$] and that from endosperm [$C_{f(endo)}$], because the DNA amounts derived from embryo and endosperm are equivalent in each seed [36]. In F1 hybrid maize having a single copy of the trait gene per maize genome, the ideal $C_{f(emb)}$ is theoretically expected to be 0.5, and on the other hand, the ideal $C_{f(endo)}$ should be 0.33 or 0.67 in paternally or maternally derived GM maize events, respectively [31]. As a result, the ideal $C_{f(seed)}$ should be 0.42 or 0.59. In this study, the experimental C_f values for trait genes in GM maize events having a single copy of transgene per genome were 0.28–0.30. The C_f values for *cp4epsps* in NK603 and MON810 × NK603 having two copies of *cp4epsps* per genome [37] were 0.72 and 0.71, respectively. In TC1507 × DAS59122 having two copies of *pat* per genome, the C_f value for *pat* was 0.61. The discrepancy between ideal and experimental values has been previously reported [2, 31] and may be due to differences in PCR efficiencies resulting from the amount of non-targeted sequences in plasmid and genomic DNA [2] or due to differences in the efficiency of DNA extraction between reference and trait genes [31].

GM maize imported to Japan contains not only single GM maize events having a single copy of the trait gene per genome, but also stacked GM maize events having multiple copies of the trait gene per genome as previously reported [25]. The corrected C_f values for each trait gene were required to calculate GMO contents in maize samples containing GM maize events having multiple copies of the trait gene per genome, because GMO content is overestimated using the C_f value calculated from each GM maize event having a single copy of the trait gene per genome. Therefore, the corrected C_f value for *cp4epsps* (0.41), which was calculated based on each C_f value in GM maize events having *cp4epsps* and the rate of a GM maize event determined from a non-IP maize sample imported from the USA in 2009, was used (Table 3). Similarly, the corrected C_f value for *pat* (0.43) was used (Table 3).

GMO content in test samples

To approximately quantitate the GMO content in five test samples (Table 1), which were prepared by mixing the ground grain samples of several GM maize and non-GM maize at 5 % (w/w) GMO content (Table 1), we extracted genomic DNA from each test sample in three parallels without degradation of DNA (Supplementary Fig. S1) and performed qPCR assays for trait- and event-specific methods (Table 5). The trait-specific method results showed lower RSD (1.3–17.1 %) as compared to those of the event-specific method (4.1–45.2 %), which is consistent with previously reported validation studies [28, 38–42]. Moreover, in the trait-specific method, the biases against theoretical values, which are calculated on the basis of the copy number of the trait gene per genome, were positive values for *cp4epsps* (3.6–18.6 %) and negative values for *pat* (−14.8 to −34.2 %). This suggests that the experimental values of GMO contents for *cp4epsps* were higher than the theoretical values, whereas those for *pat* were lower than the theoretical values. On the other hand, the absolute values of bias against theoretical value for the event-specific method were much higher (4.5–108.6 %) than those for the trait-specific method (3.6–34.2 %). In particular, the absolute values of bias in TC1507 (19.0–108.6 %) were the highest, followed by DAS59122 (50.3–63.3 %), MON863 (50.0–60.0 %), MON810 (13.0–39.9 %), NK603 (10.9–34.1 %) and MON88017 (4.5–16.5 %). These tendencies toward high biases were also shown in the previously reported validation studies [28, 38–42], where the biases were lower than those in this study. In the absolute values of total bias against theoretical value, there was little difference between the trait-specific method (1.2–6.8 %) and the event-specific method (3.6–21.8 %). These results demonstrate that the trait-specific method has higher repeatability and lower bias for each reaction, although the two methods show similar bias overall.

Test sample #1 contained only single GM maize events. Samples #2, #3 and #4 contained both single and stacked GM maize events, and the highest content of stacked GM maize events was in sample #4, followed by samples #3

Table 5 The theoretical value and experimental value (mean) of GMO content, relative standard deviation (RSD) and bias for trait- and event-specific methods

	Sample number				
	1	2	3	4	5
Trait-specific method					
cp4epsps					
Theoretical value (%)[a]	4.0	4.5	5.0	5.0	5.0
Experimental value (mean, %)	4.3	5.0	5.2	5.9	5.2
RSD (%)	13.9	1.9	13.2	17.1	11.1
Bias of experimental value against theoretical value (%)	8.5	11.9	3.6	18.6	4.1
pat					
Theoretical value (%)	0.9	1.3	1.0	2.0	2.0
Experimental value (mean, %)	0.8	1.0	0.7	1.5	1.3
RSD (%)	1.3	3.9	4.6	14.8	12.6
Bias of experimental value against theoretical value (%)	−14.8	−25.7	−25.0	−26.8	−34.2
Total					
Theoretical value (%)	4.9	5.8	6.0	7.0	7.0
Experimental value (mean, %)	5.1	6.0	5.9	7.4	6.5
RSD (%)	11.6	2.1	11.9	16.4	9.4
Bias of experimental value against theoretical value (%)	4.2	3.5	−1.2	5.6	−6.8
Bias of theoretical value against 5 % (%)[b]	−2.0	16.0	20.0	40.0	40.0
Bias of experimental value against 5 % (%)[c]	2.1	20.0	18.6	47.9	30.5
Event-specific method					
MON88017					
Theoretical value (%)	2.0	2.5	2.6	3.0	3.0
Experimental value (mean, %)	2.3	2.4	2.7	3.2	3.4
RSD (%)	20.2	45.2	10.5	17.1	19.9
Bias of experimental value against theoretical value (%)	16.5	−4.7	4.5	7.6	14.1
MON810					
Theoretical value (%)	0.9	2.1	2.5	2.5	4.0
Experimental value (mean, %)	0.5	1.4	1.8	1.9	3.5
RSD (%)	14.1	6.9	14.6	10.7	7.6
Bias of experimental value against theoretical value (%)	−39.9	−34.8	−28.9	−22.6	−13.0
NK603					
Theoretical value (%)	1.0	1.0	1.2	1.0	1.0
Experimental value (mean, %)	1.2	1.1	1.5	1.3	1.2
RSD (%)	7.3	8.1	10.3	4.4	5.2
Bias of experimental value against theoretical value (%)	16.0	10.9	21.9	34.1	20.2
TC1507					
Theoretical value (%)	0.6	0.7	0.5	1.0	1.0
Experimental value (mean, %)	1.3	0.8	0.3	0.7	0.7
RSD (%)	44.0	18.4	6.9	7.1	8.0
Bias of experimental value against theoretical value (%)	108.6	19.0	−33.2	−27.8	−28.5
DAS59122					
Theoretical value (%)	0.3	0.6	0.5	1.0	1.0
Experimental value (mean, %)	0.5	0.9	0.8	1.6	1.6
RSD (%)	9.8	4.1	10.3	7.2	8.3
Bias of experimental value against theoretical value (%)	63.2	51.2	50.3	56.8	63.3
MON863					
Theoretical value (%)	0.2	0.1	0.0	0.0	0.0
Experimental value (mean, %)	0.3	0.2	0.0	0.0	0.0
RSD (%)	17.5	4.7	–	–	–

Table 5 continued

	Sample number				
	1	2	3	4	5
Bias of experimental value against theoretical value (%)	60.0	50.0	–	–	–
Total					
Theoretical value (%)	5.0	7.0	7.3	8.5	10.0
Experimental value (mean, %)	6.1	6.7	7.0	8.8	10.5
RSD (%)	4.8	14.8	4.3	4.9	4.2
Bias of experimental value against theoretical value (%)	21.8	−3.6	−3.5	3.4	4.5
Bias of theoretical value against 5 % (%)	0.0	40.0	46.0	70.0	100.0
Bias of experimental value against 5 % (%)	21.8	35.0	40.8	75.9	109.1

[a] This value is calculated based on the copy number of transgene per genome

[b] Bias of theoretical value against 5 % GMO content on a w/w basis

[c] Bias of experimental value against 5 % GMO content on a w/w basis

and #2. Test sample #5 contained only stacked GM maize events. In regard to bias of the experimental value against 5 % (w/w) GMO content, the trait-specific method (2.1–47.9 %) showed lower bias than the event-specific method (21.8–109.1 %); specifically, the difference in bias between the two methods was largest in test sample #5 (30.5 and 109.1 % for trait- and event-specific methods, respectively). These results suggest that the overestimation of the event-specific method was higher than that of the trait-specific method in proportion to the content of the stacked GM maize event. Moreover, these results are consistent with the bias of theoretical value against 5 % (w/w) GMO content (−2.0–40.0 % for the trait-specific method and 0–100.0 % for the event-specific method). These results suggest that the difference in bias between the two methods was attributed not only to the accuracy of the method, resulting from the difference in calibrant, PCR efficiency and so on, but also to the principle of the method. The uncertainty in GMO content determined by the trait- and event-specific methods of the sample containing stacked GM maize events is largely attributed to sample characteristics, such as the content and the kind of stacked GM maize events. Theoretically, the uncertainty in GMO content determined by the trait-specific method is expected to be smaller than that determined by the event-specific method, because the overestimation of the trait-specific method is smaller than that of the event-specific method. This theory was substantiated by this study. Each test sample was designed according to the distribution of GM maize events in the US market in the past, present and future. Test sample #1, containing only single GM maize events, was modeled on GM maize events planted in the past. Test samples #2, #3 and #4, containing single and stacked GM maize grains, were modeled on GM maize events planted in the present, and sample #5, containing only stacked GM maize grains, was modeled on GM maize events to be planted in the future. The results of this study suggest that the trait-specific method has higher trueness (GMO content on a w/w basis) than the event-specific method, especially for the GM maize events planted in the present and future.

A drawback of the trait-specific method, if any, is the possible oversight of single GM maize events having no *cp4epsps* and *pat*, such as MON810 and MON863 (Supplementary Table S1). The National Agricultural Statistics Service (NASS) reported that the percentage of stacked GM maize events has consecutively increased from 1 % in 2000 to 71 % in 2013 in the USA [43]. Indeed, our previous studies showed that the percentage of stacked GM maize events in non-IP samples imported to Japan from the USA increased from 12 % in 2005 [24] to 35 % in 2009 [25]. Judging from these data, almost all GM maize is composed of stacked GM maize events, indicating that no single GM maize events will be distributed commercially in the future. In this situation, the potential oversight of single GM events having no *cp4epsps* and *pat* can be ignored. The content of single GM maize events having no *cp4epsps* and *pat* would be measured from non-IP maize samples imported into japan in future using the individual kernel detection method [15]. Furthermore, the corrected C_f for *cp4epsps* or *pat* would need to be consecutively updated by monitoring the GM maize events in non-IP maize samples imported into Japan, because the rates of the GM maize events would be predicted to vary every year. In addition, the trait-specific method can prove GMO content, but not the existence of stacked GM maize events in maize sample, whereas the individual kernel detection method enables identification of stacked GM maize event in a maize kernel by combining with the event-specific method [17, 18, 44].

In countries evaluating GMO content on a w/w basis, current event-specific method has the potential to lead to an excess of the regulatory threshold levels of GMO content in the ground maize samples containing stacked GM maize events, even though the actual GMO content is lower than the regulatory threshold levels. We believe that the proposed trait-specific method would be the only feasible way to solve this problem and would be useful not only for the countries importing maize and requiring the verification of the labeling regulation on a w/w basis, such as Japan and Korea, but also for the countries exporting maize, such as the USA and Brazil.

Conclusion

In this study, we developed a trait-specific method that can quantitate GMO content by measuring only *cp4epsps* and *pat* using qPCR. This method overcomes the drawback associated with event-specific methods, in which the GMO content of stacked GM maize samples is greatly overestimated. The developed trait-specific method would be the only feasible way to conduct the quantification of GMO content in the ground maize samples containing stacked GM maize, which will increasingly be found in the future, for the verification of the labeling regulation.

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Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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第2章　第8節　iPS・幹細胞の培養を成功させる技術

［4］ ニワトリの胚性幹細胞研究と培養技術

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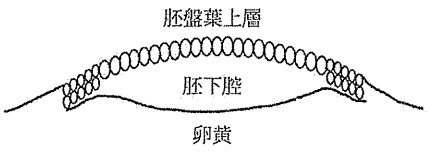
第8節 [4] ニワトリの胚性幹細胞研究と培養技術

はじめに

マウスや霊長類では，胚盤胞の内部細胞塊から胚性幹（embryonic stem，ES）細胞が樹立されている。しかし，鳥類であるニワトリは，卵生で胎盤が形成されないため，発生初期において内部細胞塊に相当する細胞は見られない。そこでニワトリでは，Eyal-Giladi and Kochav [1] の発生ステージ（I～XIV）のステージXの胚盤葉細胞が利用されている。これは，このステージの胚盤葉細胞の移植によって作製されたキメラ体が生殖系列キメラとなることが確認されたことによる [2]。その後，ニワトリでは1996年に胚盤葉細胞からES細胞が樹立されたが [3]，生殖系列キメラが確認されたのは，7日間培養した胚盤葉細胞であった [3]。この胚盤葉細胞の培養では，基本的にマウスES細胞の培養系が参考にされており，特にマウスのES細胞が *in vitro* で多能性を維持するために必要なマウス由来白血病阻害因子（leukemia inhibitory factor，LIF）が細胞増殖にも未分化マーカーの発現にも効果が高いことが報告されている [3]。筆者のグループは，これまで行ってきた鳥類免疫系サイトカインの分子生物学的な解析から，ニワトリの免疫系サイトカインは，ほ乳動物のそれとアミノ酸レベルでの同一性が極めて低いことを認識していた。そこで，胚盤葉細胞の培養には，ニワトリ由来のLIFを用いた方がより効果的ではないかと考えた。実際にニワトリのLIF遺伝子をクローニングしたところ，予想通りヒトやマウスのLIFとアミノ酸レベルで40％前後の同一性しかないことがわかった [4]。興味深いことに，胚盤葉細胞に対するLIFのシグナル伝達能をニワトリとマウスのLIFで比較したところ，細胞増殖のシグナルとして機能するMEKのリン酸化は，ニワトリでもマウスでも一様に起きるのに対して，多能性の維持に機能するSTAT3のリン酸化は，ニワトリLIFでのみ起きることがわかった [4]。さらに胚盤葉細胞におけるニワトリLIFのSTAT3のリン酸化を抑制すると，胚盤葉細胞が分化してしまうこと，培養初期の胚盤葉細胞はLIFを産生し，オートクリンで多能性を維持している可能性があることも明らかになった [5]。著者のグループでは，ニワトリLIFをニワトリ細胞に産生させ，このLIFと継代方法を工夫することで，これまでに長期継代培養が可能な100種以上の培養胚盤葉細胞を樹立している。またこれらの細胞は，支持細胞存在下で霊長類ES細胞様のコロニーを形成すること，多能性に寄与する転写因子であるNanogと生殖細胞特異的因子であるVasaを発現していること，また移植胚において高頻度に生殖系列キメラに寄与するなどの結果も得ている [6]。ニワトリES細胞の由来は，ステージXの胚盤葉であり，この時期の胚盤葉は胚盤葉上層（epiblast）からなる。即ち，ニワトリES細胞はその性状からembryonic stem cellではあるが，正確には細胞の由来からepiblast-derived stem cellである。本項では，ニワトリ胚盤葉細胞の培養方法について概説する。

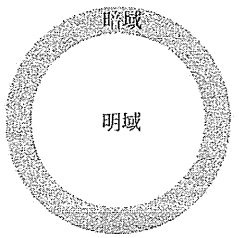
1. 胚盤葉細胞の分離方法

ステージXの胚盤葉上層（epiblast）は，受精後，子宮内（卵管内）で20時間が経過しており，約6万個の細胞からなる（図1）。胚盤葉上層は，細胞の脱落により中央域で単層上皮性の細胞層となっており，この領域は明るく透明に見えるため明域と呼ばれ，胚壁に由来する部分を暗域と呼ぶ（図2）。明域には，Vasa陽性細胞が数十個確認されており [7]，このVasa陽性細胞が始原生殖細胞から生殖細胞に分化すると考えられている。そのため，生殖系列キメラを



ステージXの胚盤葉上層（断面）

図1 ステージXの胚盤葉を断面で見た場合の模式図



ステージXの胚盤葉上層の明域と暗域

図2 ステージXの胚盤葉を上部から見た場合の模式図

意識した実験系では，胚盤葉上層を受精卵から回収した後，明域のみをさらに分離する手法がとられる。ここでは，簡便な胚盤葉上層を形成する胚盤葉細胞の単離方法を記述する。

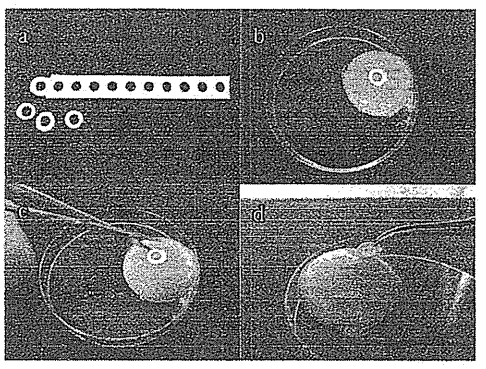
1.1 受精卵

使用する受精卵は，可能な限り産卵直後の新鮮なものを使用する。ニワトリ胚の発生は28℃以上で進行するが，産卵後の時間と環境温度で胚盤葉下層の形成が始まるので注意する。

1.2 胚盤葉上層の分離

胚盤葉上層の分離には，PBS（phosphate buffered saline），直径10 cmのプラスチック製シャーレ（無菌），ステンレス製のハサミ（小直剪刀両鋭），ピンセット，ろ紙，穴あけパンチを用いる。予めPBSはオートクレイブ，ハサミとピンセットは乾熱滅菌をしておく。ろ紙は，穴あけパンチ（スライド多穴式が便利）で直径5 mmの穴を開け，その外輪に沿ってハサミで円形にカットし，ろ紙のリングを作製する（図3a）。ろ紙のリングは，オートクレイブで滅菌し，乾燥させておく。

受精卵は，エッグセパレーター等を用いて卵白を完全に除去したのち，プラスチック製シャーレ中に静置する。この際，胚盤葉が卵黄の上部に位置するように留意する。滅菌乾燥させたろ紙のリングを中央に胚盤葉が位置するように張りつける（図3b）。ろ紙のリングの外縁に沿ってハサミ（小直剪刀両鋭）を入れ（図3c），卵黄膜ごと円形にカットする。ピンセットでろ紙のリングをつまみ上げる。つまみ上げる際，つまんだ端からろ紙をゆっくり斜めに持ち上げることで，付着する卵黄を可能な限り除去する（図3d）。このとき，ろ紙のリングには，胚盤葉上層が張り付いた状態となる。卵黄側を上にして（ひっくり返す），滅菌PBS入りのシャーレに浸し，ゆっくりろ紙のリングを揺すって，付着した卵黄を除去する。別に準備した滅菌PBS入りシャーレにろ紙のリングを移し，少しだけ激しく揺することで，胚盤葉上層の細胞がろ紙のリングから円盤状に分離する。分離した胚盤葉上層の細胞は，マイクロピペットで1.5 mLのチューブに回収する。



a：ろ紙で作製したリング　b：ろ紙を卵黄膜に貼付ける。
c：ろ紙の外枠に沿って卵黄膜をカットする。
d：ろ紙をピンセットで静かに持ち上げる。斜めの状態を保ち卵黄を除去する。

図3　ステージXの胚盤葉上層の分離方法

2. 分離した胚盤葉上層の培養方法

分離した胚盤葉上層の細胞は，事前に準備しておいた支持細胞上で培養する。支持細胞には，ニワトリ胚線維芽細胞（CEF），マウス胚線維芽細胞（MEF）やマウス胚線維芽細胞由来細胞株（STO）などが利用できる。いずれの細胞もイラジエーションやマイトマイシンC処理により，細胞増殖を停止させておく必要がある。これまでのところ，ニワトリ胚盤葉細胞もしくはそこから樹立したES細胞の無支持細胞培養系は確立されていない。著書のグループでは，樹立したES細胞の核型解析や多能性や生殖細胞分化に機能する遺伝子の発現を試験する必要性から，異種動物由来であるSTO細胞を汎用している。

2.1 支持細胞のワーキングストックの準備

著書のグループでは，前述の通り支持細胞にSTO細胞を利用している。STO細胞はATCC（American type culture collection）や種々の業者から導入することができる。導入したSTO細胞は，最初の培養時に多量の凍結保存細胞を準備しておき，そこからマイトマイシンC処理したワーキングストックを準備している。これはたとえ株化された細胞株であっても継代回数を重ねることで細胞の性状変化が起こることを懸念しての対処である。ワーキングストックは，

常に同じ継代数のSTO細胞で利用できるようにしている。以下に一例としてワーキングストックの調整例を記述する。

最初の凍結保存STO細胞は，溶解後，直径10 cmの培養用シャーレ2枚に播種する。培養液は，10% FBS（fetal bovine serum）-DMEM（Dulbecco's modified Eagle medium）を使用し，培養条件は，5% CO_2，37℃の条件下で行う。STO細胞は，約3日間の培養でコンフルエントに達し，冷PBSで3回洗浄した後，0.025% トリプシン，0.02% EDTA 2Na-PBSで細胞を剥離し，これを直径15 cmの培養用シャーレ8枚に播種する。細胞がコンフルエントに達したら，培養液にマイトマイシンCを終濃度で10 μg/mLとなるように加え2時間培養する。冷PBSで5回洗浄し，0.025% トリプシン，0.02% EDTA 2Na-PBSで細胞を剥離し，遠心洗浄を少なくとも3回行う。血球計算盤を用いて細胞数を算出する。ES細胞やiPS細胞の培養には，ハンドリングの良さから直径6 cmの培養用シャーレが利用されており，支持細胞はその培養有効面積に応じて調整されている。例えば，マウスES細胞の場合は7-8 × 10^5 cells/6 cm dishであり，ヒトES細胞の場合は，3-4 × 10^5 cells/6 cm dishである。ニワトリの胚盤葉細胞の培養の場合，著書のグループのこれまでの培養実績から2-3 × 10^5 cells/6 cm dishの濃度の支持細胞を使用している。この条件で8枚の直径15 cmの培養用シャーレからワーキングストックを準備すると，300 ～ 400枚分の6 cmの培養用シャーレ用ワーキングストックが準備できる。著書のグループでは，一本分の凍結保存用チューブに6 cm dishで10枚分のワーキングストックを準備している。

2.2 支持細胞の準備

支持細胞の培養には，他種のES細胞やiPS細胞と同様，培養用シャーレのゼラチンコートがニワトリ胚盤葉細胞の培養にも有効である。ゼラチンコート液は，ゼラチンを0.1%になるように蒸留水に添加後，オートクレイブにより溶解，滅菌したものを使用する。培養dishは，支持細胞を培養する少なくとも2時間前に，底面がゼラチンコート液に浸る状態とし37℃でコートする。ゼラチンコート液を除去後，2-3 × 10^5 cells/6 cm dishの濃度のマイトマイシンC処理済みの支持細胞を10% FBS-DMEMに調整して播種する。支持細胞は，播種後翌日から使用可能であり，5日以内に使用する。

2.3 胚盤葉上層細胞の初代培養

ひとつの胚から分離した胚盤葉上層の細胞は，一枚の支持細胞を播種した6 cm dishで培養を開始する。胚盤葉細胞の培養に使用する培地（chicken ES medium，CESM）の組成は表1に示した。ニワトリLIFは，37℃に加温しておいたCESMに使用直前に20 ng/mLの濃度になるように添加する。1.2で回収した胚盤葉上層は，CESMでピペッティングすることで簡単に分散できる。分散後，CESMで3回遠心洗浄する。支持細胞は，加温したCESMで3回洗浄し，遠心洗浄した胚盤葉上層細胞を，LIFを添加したCESMに再浮遊させ播種する。著書のグループでは，上記の播種した細胞を5% CO_2，3% O_2，38℃の条件で培養している。培養1日後に加温したLIF添加CESMで培地の半量交換を行う。培養開始後2日後には，図4に示したように支持細胞を押しのけるように増殖した胚盤葉細胞のコロニーが観察される。これ以降，継代後も培地は毎日，LIFを添加したCESMで半量交換を行う。

表1 chicken ES medium（CESM）の組成

試薬名（メーカー，カタログ番号#）	最終濃度
KnockOut Serum Replacement（Gibco #10828-028）	20%
chicken serum（Gibco #16110-082）	2%
sodium pyruvate（Gibco #11360-070）	1%
MEM NEAA（Gibco #11140-050）	1%
GlutaMax（Gibco #35050-061）	1%
100X nucleosides（Millipore #ES-008D）	1%
Antibiotic-Antimycotic mixed stock solution（nacalai tesque #09366-44）	1%
β-mercaptoethanol（Sigma #M7522）	0.1 mM
KnockOut-DMEM（Gibco #10829-018）*1	
recombinant chicken LIF*2	20 ng/mL

*1 使用する基礎培地
*2 recombinant chicken LIFは，加温した最低限必要な量の培地に使用直前に添加する。

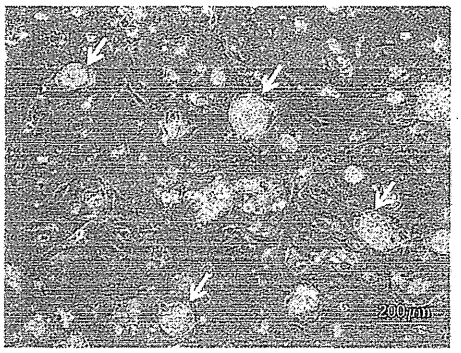


図4　培養開始2日後の胚盤葉細胞のコロニー（矢印）

2.4　胚盤葉上層細胞の継代培養

初代培養3～5日目には，胚盤葉細胞のコロニーの直径が約1mmになるまで増殖し，継代を行うタイミングになる。培養胚盤葉細胞は，マウスのepiblast-derived stem cellや霊長類ES細胞と同様にプロテアーゼを用いて分散させると細胞増殖が停止してしまう。そのため，培養胚盤葉細胞の継代では，トリプシンなどのプロテアーゼを使用せずに，物理的にコロニーを小さく砕くようにして行っている。その方法には，StemPro EZPassage（Invitrogen）などを用いる方法やマイクロピペットに付けたチップの先でコロニーを分散させる方法がある。どちらの手法も，若干のテクニックが必要である。継代1回目以降，細胞は旺盛に増殖するため2～3日置きに継代する必要がある。また，形成されるコロニーの中には，図5に示したように黒ずんでくる（顕微鏡下の観察では，茶色に見える）ものが出現する。このコロニーは増殖せず，また他の増殖中の細胞に悪影響を及ぼすため，実体顕微鏡下で継代時にマイクロピペットで削り取り除去しておく。以上の条件で通常は，3～4回の継代回数，培養日数では10～20日の培養が可能である。この時，多くの細胞は，黒ずむコロニーを形成するものが大半を占めるようになり，継代培養が困難となる。しかし，分離した胚盤葉上層の細胞によっては，黒ずむコロニーが無くなり，安定して増殖するようになる（図6）。現在のところ，その原因についてはわかっていない。安定して増殖するようになった胚盤葉上層の細胞は，初代培養の細胞と同様にNanogやVasaの発現が維持されている。著書のグループでは，安定して増殖するようになったこれらの胚盤葉上層の細胞をES細胞と呼び，核型解析も行っている。その結果，ES細胞では2～10%の割合で染色体の異常が認められる。このようにして樹立したニワトリES細胞を初期胚に移植したところ，培養40日のES細胞から生殖系列キメラが得られ，後代検定によってES細胞由来の後代が得られている。

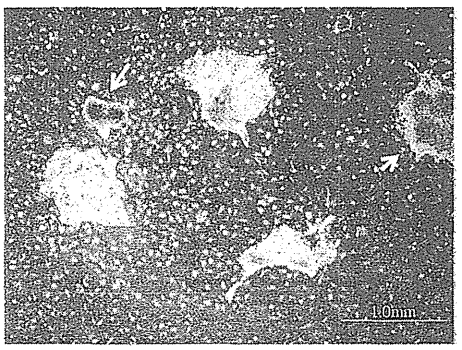


図5　増殖を停止したコロニー（矢印）

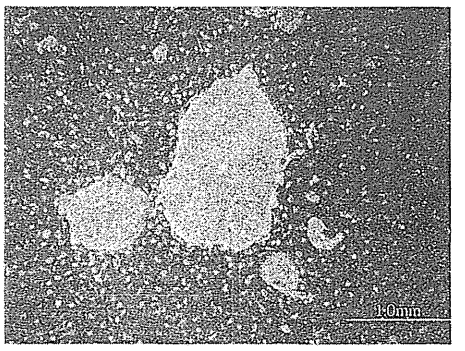


図6　安定に増殖する胚盤葉細胞のコロニー