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国内情報

# わが国でこれまでに生産された体細胞クローン牛およびその後代牛の状況

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## 1. はじめに

体細胞クローン牛の生産動向については、農林水産省が国内で生産された体細胞クローンの生産状況と転帰を調査し、「異動報告」を毎月、「現状」を半年ごとに、それぞれ、定期的にプレスリリースしている。これによって、死産、生後直死あるいは病死などの発生状況に関しては、農林水産省のHPから常に最新で正確な情報を得ることができる（http://www.s.affrc.go.jp/docs/press/101224.htm）。図1に平成22年9月30日現在の最新データを示す。これによると、これまで生産された588頭の体細胞クローン牛のうち、死産は84頭（14%）、生後直死は94頭（16%）ならびに病死は148頭（25%）であることがわかる。このデータに基づき、説明者が体細胞クローン牛の生産動向の現状を的確に把握した上で、質問者に正確な情報を提供することができる。しかし、「このデータを一般牛（非クローン牛）と比較した場合どうなのか」と質問者に問われると、説明者は答えに窮する。結局、牛を含む家畜の場合、一般動物に関する上記データの統計は存在しないようである。

生産動向とならんで質問者が関心を示す情報は、体細胞クローン牛の寿命である。たとえば、体細胞クローンに対するコメントや書き込みなどをみると、「テロメアが短い（老化が進んでいる）」、「死亡率が高い」、「長生きできない」という記載が多々認められる。実際、（独）農研機構 畜産草地研究所を訪れた体細胞クローン牛の見学者の質問にも、これらの類の話題が飛び出す頻度は高い。これらの知識は、たとえば、「体細胞クローン羊・ドリーの早世」などの報道に基づくものと思われる。これらの報道はある動物個体で認められた事実であるとし

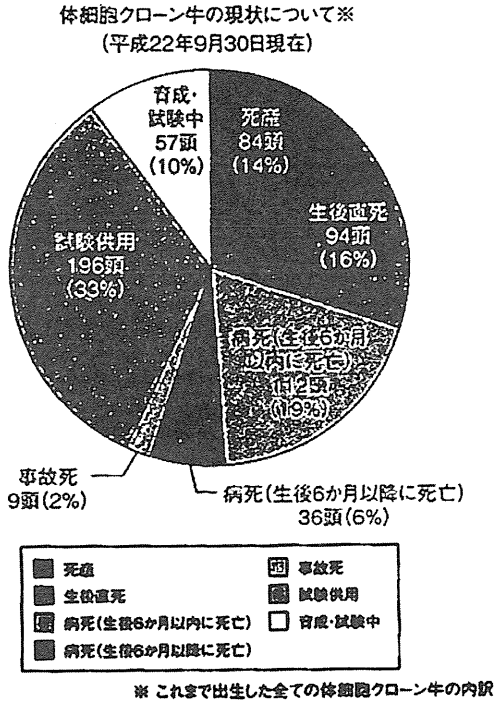


図1 体細胞クローン牛の生産状況
（農林水産省のHP：http://www.s.affrc.go.jp/docs/press/101224.htmより）

ても、「『クローン家畜』の集団」としても一般的に認められる現象なのかどうかは不明確である。

クローン家畜の寿命を説明するためには、一般家畜の寿命を把握しておく必要がある。しかし、家畜の寿命に関する統計データは極めて少ない。なぜならば、大部分の家畜では、人間の食料生産に用いられるため、寿命を全うすることがないからである。

家畜の寿命に関連する数少ない統計データとして、容易に入手できるものとして、独立行政法人家畜改良センター（以下、改良センター）による「牛の個体識別情報検索サービス」の「届け出情報の統計」がある（https://www.id.nlbc.go.jp/data/toukei.html）。その統計データによって、死亡牛の数とその時の月齢やと畜牛の数とその時の月齢を知ることができる（図2）。しかしながら、牛では、前述のように食用に供するためにと畜される場合が多いため、自然死を迎える個体は極めて少ない関係上、たとえば、「牛の寿命は何歳ですか」という質問者を満足させる答えをこれらの統計データの中から説明者が見い出すことは困難である。

このように、改良センターの貴重なデータを駆使しても、「体細胞クローン牛の寿命は短いと聞いていますよ。実際、ドリーは早死にしているし…」という質問者からの問いかけに対しても、説明者が明確な回答を提供することも困難である。

これらの説明上の悩みを解消すると同時に、国内で生産された体細胞クローン牛およびその後代牛の出生状況やその後の転帰に関する客観的データを収集するため、2006（平成18）年8月、体細胞クローン牛の生産実績のある機関に対する調査を実施した。本稿では、この調査のうち、体細胞クローン牛とその後代牛の死亡状況に関する部分を紹介する。

## 2. 今回紹介する情報の基となっている調査データ

調査への協力を依頼した結果、39機関から、体細胞クローン牛：482頭、後代牛：202頭（27機関で生産）のデータが寄せられた。調査当時のわが国では、495頭の体細胞クローン牛が生産されていた（平成18年3月現在の農林水

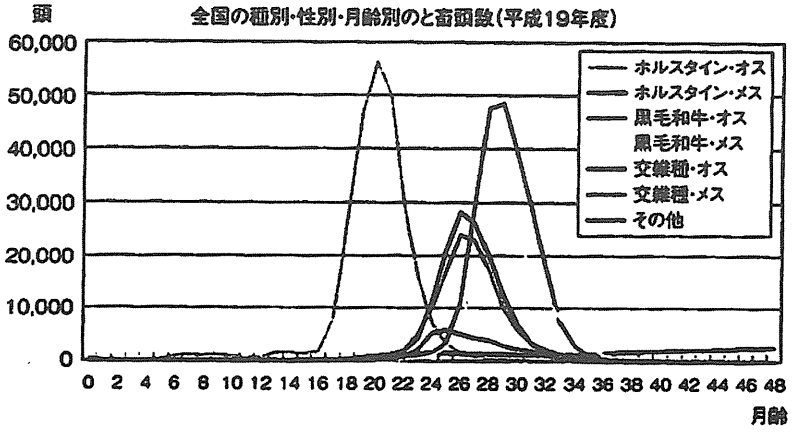


図2（独）家畜改良センターにより公表されていると畜された牛に関するデータ
（同センターのHP：https://www.id.nlbc.go.jp/data/toukei.html より）

産省プレスリースによる）ことから、この調査時点までに生産されていた体細胞クローン牛の97.3%に相当するデータが収集できたことになる。これらのデータのうち、一般牛のデータ(国内の3牛群）が収集できたホルスタイン種と黒毛和種の体細胞クローン牛とその後代牛のデータを対象に、これらの統計的有意性を以下に検証していく。

## 3. 死産・生後直死の発生状況

これらの検証に用いた牛は、ホルスタイン種と黒毛和種の体細胞クローン牛（451頭）、後代牛（124頭）および一般牛（566頭）である。

死産の発生率は、体細胞クローン牛：16.4%（74/451）、後代牛：8.9 %（11/124）および一般牛：4.6%（26/566）である。有意差は、体細胞クローン牛と後代牛との間（P<0.05）、体細胞クローン牛と一般牛との間（P<0.01）で認められる。一方、後代牛と一般牛との間で有意性は認められない（図3）。調査表に死因が記載されていた症例において、死産のうち、20.8%（10/48）が難産、また、16.7%（8/48）が窒息、羊水誤嚥などの呼吸障害による死亡と記録されている。

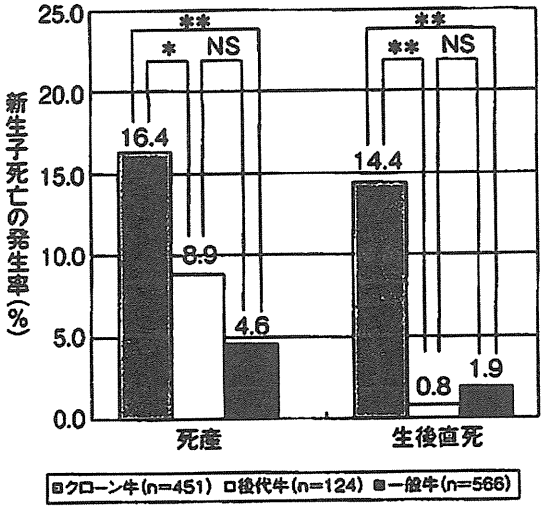


**図3 体細胞クローン牛、後代牛及び一般牛における死産と生後直死（ホルスタイン種・雌、黒毛和種・雌雄）**

注1) これまでに生産されたホルスタイン種の体細胞クローン牛は全て雌なので、対照となる後代牛や一般牛も全てホルスタイン種の雌を用いている。

注2) *：P<0.05、**：P<0.01、NS：有意差なし（$\chi^2$検定による）。

生後直死の発生率は、体細胞クローン牛：14.4%（65/451）、後代牛：0.8%（1/124）および一般牛：1.9%（11/566）である。有意差は、体細胞クローン牛と後代牛との間（P<0.01）、体細胞クローン牛と一般牛との間（P<0.01）で認められる。しかし、後代牛と一般牛との間で有意性は認められない（図3）。調査表に死因が記載されていた症例において、生後直死のうち、50.7%（35/69）が窒息、羊水誤えんなどの呼吸障害による死亡である。

生後直死した体細胞クローン牛では、過大子の傾向が認められる。たとえば、ホルスタイン種・雌の体細胞クローン牛における生時体重は、生後直死の牛：53.6±11.2（n=16、平均±SD、以下同様）、生存中の牛：44.5±10.4kg（n=9）である。一方、同時期の一般牛群（ホルスタイン種・雌）の生時体重は、40.5±5.8kg（n=137）である。なお、3例分しか生後直死のデータが集まらなかった後代牛については、統計的な比較はできない。

## 4. 病死の発生

この検証に用いた牛は、ホルスタイン種と黒毛和種の体細胞クローン牛（216頭）、後代牛（64頭）および一般牛（991頭）である。

検証に際しては、1カ月ごとの期間に区切り、病死頭数をその期間当初の生存頭数で割って求めた病死率を集計している。その結果、体細胞クローン牛の病死率は、生後200日齢頃まで一般牛よりも高い傾向を示すが、それ以降

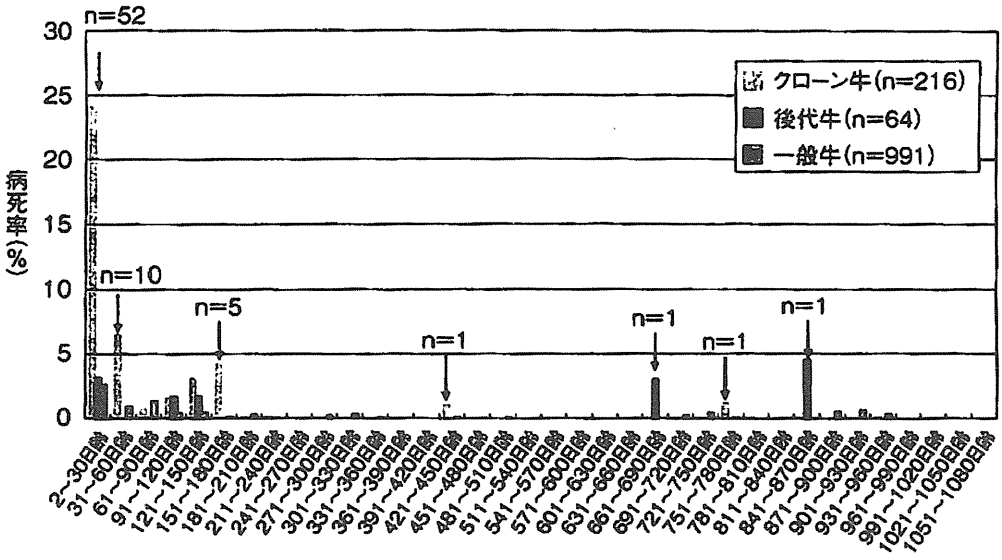


図4 体細胞クローン牛、後代牛及び一般牛における病死率の推移（ホルスタイン種・雌及び黒毛和種・雌雄、死産と生後直死を除く）

注1) 図1と同じ母集団（ホルスタイン種・雌、黒毛和種・雌雄）のデータの中から試験とさつを除く。また、これまでに生産されたホルスタイン種の体細胞クローン牛は全て雌なので、対照となる後代牛や一般牛も全てホルスタイン種の雌を用いている。

注2) 1カ月ごとの期間に区切り、その期間の「病死率＝病死頭数／生存頭数×100」をプロットする。

は一般牛同様に極めて低いレベルで推移すること、後代牛においては生後2日目以降、一般牛とほぼ同等の死亡率で推移している（図4）。調査表に死因が記載されていた症例における生後2～3日の死因は、窒息などの呼吸障害（6/17）や心臓奇形（2/17）が多い。それ以降の病死は肺炎によるものが最も多い。後代牛では死因の記録が残っていない症例が多いため、死因の傾向を明らかにすることは困難な状態である。

次に、体細胞クローン牛、後代牛および一般牛における病死率を2～150、150～300および300～720の時期ごとに区切って比較した結果、2～150日齢の病死率は、体細胞クローン牛：23.5%（72/307）、後代牛：4.5%（5/111）および一般牛：4.3%（55/1289）である（表5）。有意差は、体細胞クローン牛と後代牛との間（$p<0.01$）、体細胞クローン牛と一般牛

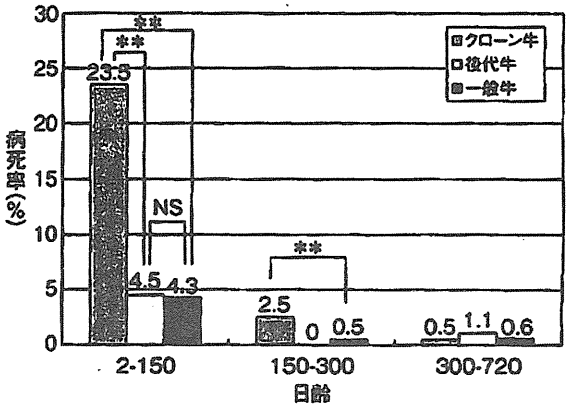


図5 体細胞クローン牛、後代牛および一般牛における各時期の病死率（ホルスタイン種・雌及び黒毛和種・雌雄）

注1) 図10と同じ母集団（ホルスタイン種・雌、黒毛和種・雌雄）のデータに基づく。

注2) ＊：$P<0.05$、＊＊：$P<0.01$、NS:有意差なし（$\chi$自乗検定による）。

との間（p<0.01）で認められるが、後代牛と一般牛との間で有意性は認められない。150～300日齢の病死率は、体細胞クローン牛：2.5%（5/202）、後代牛：0%（0/94）および一般牛：0.5%（6/1207）である。有意差は、体細胞クローン牛と一般牛の間（p<0.01）で認められる。また、300～720日齢の病死率は、体細胞クローン牛：0.5%（1/185）、後代牛：1.1%（1/88）および一般牛：0.6%（6/1089）で、これらはほぼ同等の水準である。

## 5. 体細胞クローン牛およびその後代牛における死亡状況のまとめ

体細胞クローン牛では、死産、生後直死や幼若期の病死率が一般牛よりも高いが、生後200日齢頃までに一般牛と同水準の病死率になると考えられる。したがって、黒毛和種における肥育もと牛の出荷（約10カ月（300日）齢）や食肉出荷（24カ月（720日）齢以降）、ホルスタイン種雌の初産分娩（24カ月（720日）齢以降）の時期に生存している体細胞クローン牛の強健性は一般牛と同等と考えられる。一方、後代牛における死産、生後直死および病死の発生率は、調査した全期間で一般牛との有意な差異は認められない。

**謝辞**

この調査に際して、多大なご協力をいただいた全国39機関の関係各位に深甚なる謝意を表します。

**参考文献**

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RAPID COMMUNICATION

# Survival of embryos and calves derived from somatic cell nuclear transfer in cattle: a nationwide survey in Japan

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## ABSTRACT

To obtain data concerning the survival of embryos and calves derived from somatic cell nuclear transfer (SCNT) in Japan, a nationwide survey was carried out in April, 2009. As a result, data concerning 3264 embryo transfers (ETs) with SCNT embryos which produced 301 calves were accumulated and their survival was analyzed. The present survey revealed that survival rates of transferred bovine embryos and produced calves derived from SCNT had not improved over a decade (1998–2007). A remarkable feature of the pregnancies with SCNT embryos was a high incidence of spontaneous abortions. When the decade was divided by the occurrence of bovine spongiform encephalopathy (BSE) in 2001, significant decreases in the 'after BSE' period (2002–2007) were observed in the percentages of calves born ($P < 0.01$), calves living at birth ($P < 0.05$), calves living for 24 h ($P < 0.05$) and 6 months ($P < 0.01$). Abortions that occurred during 61–99 days after ETs were significantly increased ($P < 0.01$) in the 'after BSE' period. Certain kinds of regeneration that occurred in oocytes during the 15–20 h of storage of bovine ovaries at 10–15°C as a part of BSE inspection might have had some negative effects on SCNT embryos when these oocytes were used as recipients of SCNT.

***Key words:*** *calf, cattle, embryo, somatic cell nuclear transfer, survival.*

## INTRODUCTION

Somatic cell nuclear transfer (SCNT) technology has been considered as a promising innovatory technology for livestock agriculture (Wells 2003, 2005; Bousquet & Blondin 2004; Campbell *et al.* 2007), endangered animal conservation (Wells 2005) and human medicine (Wells 2005; Campbell *et al.* 2007). For the practical use of SCNT technology in livestock agriculture, a risk assessment of the food safety of cloned animals is required for every country or region.

The first risk assessment on the food safety of SCNT animals was released by the Food and Drug Administration (FDA) of the USA in January, 2008 (FDA 2008). With regard to Japan, the Food Safety Commission (FSC) of Japan issued a risk assessment report concerning the food safety of SCNT cattle/pigs and their progeny in July, 2009 (FSC 2009). The report of the FSC, which was the first risk assessment report concerning foods derived from SCNT animals and their progeny in Asian – Australasian countries, concluded that food products derived from SCNT cattle, pigs and their progeny have substantial equivalence to cattle and pigs produced by conventional assisted reproduction technologies (ARTs) such as artificial insemination (AI) and embryo transfer (ET). Facing this assessment, the Ministry of Health, Labour and Welfare of Japan, which is in charge of food sanitation laws, concluded that the law shall not regulate the foods derived from SCNT animals and their progeny. However, in August, 2009, the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan issued a notification demanding a continuation of the voluntary moratorium since 1999 owing to the low efficiency of animal SCNT and the lack of consumer acceptance toward clones.

The notification suggests that improving the efficiency of animal SCNT is required for the practical use of the technology in livestock agriculture. Considering its practical use, examples of large-scale ETs with SCNT embryos would be essential as model cases of the commercial use of the technology. Such examples were undertaken in the USA (Pace *et al.* 2002; Panarace *et al.* 2007) and Argentina (Panarace *et al.* 2007);

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however, there have been no examples in Japan. Therefore, a nationwide survey concerning ETs with SCNT embryos and produced calves in Japan was conducted and the large-scale data that were obtained were analyzed to clarify the status of SCNT technology over a decade (1998–2007).

## MATERIALS AND METHODS

### Nationwide survey in Japan

A nationwide survey on ETs with SCNT embryos and produced calves was carried out in April, 2009. Data sheets were sent to Japanese institutions that have produced cloned cattle and 21 institutions submitted their data. The data referred to 3264 ETs with SCNT embryos and 301 produced calves (as of December 31, 2007 for ETs). All ETs with bovine SCNT embryos were carried out with one embryo per recipient, since it has been believed that the transfer system should be effective for giving relief to dystocia of recipients caused by large offspring syndrome (LOS). According to a press release by MAFF (http://www.s.affrc.go.jp/docs/press/press.htm), the 301 cattle represent 54.6% (301/551) of all the SCNT cattle produced during the period subject to this survey.

### Data analysis

With the nationwide data, parameters concerning survival of transferred SCNT embryos and produced calves were analyzed. Nationwide statistics of calf production using 'multiple ovulation and embryo transfer (MOET)' and '*in vitro* production of embryos (IVP)' collected by MAFF (MAFF data; http://www.maff.go.jp/j/chikusan/sinko/lin/l_katiku/pdf/h19kekka.pdf) were used for comparison of the technological level. Statistical analysis was carried out using $\chi^2$ test for the parameters.

## RESULTS AND DISCUSSION

### Survival of SCNT embryos to term

The present survey revealed that survival of transferred bovine embryos and calves derived from SCNT had not improved over a decade (1998–2007) (Table 1). The survival levels of SCNT embryos and produced calves were similar to those in other large-scale observations in cattle (Pace *et al.* 2002; Panarace *et al.* 2007). When the pregnancy rates of ETs with SCNT embryos were compared with those of MOET and IVP in MAFF data, the result over the decade of SCNT (21.6–32.9%) was lower than those of MOET (50–52%) and IVP (37–46%) (Fig. 1). With regard to calving rates, the result of SCNT embryos over the decade (5.1–12.6%) was also lower than those of MOET (23.9–34.8%) and IVP (20.2–22.3) (Fig. 2). These findings show that the survival rates of transferred bovine embryos and produced calves derived from SCNT were lower than those of MOET and IVP owing to a low pregnancy rate and a high frequency of abortions. As reported elsewhere (Pace *et al.*

**Table 1** Survivals of transferred bovine embryos and produced calves derived from somatic cell nuclear transfer over a decade (1998–2007)

| | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| No. of SCNT† embryos transferred | 253 | 418 | 428 | 435 | 403 | 411 | 337 | 324 | 149 | 106 | 3264 |
| No. (%) of pregnant recipients‡ | 62 (24.5) | 111 (26.6) | 100 (23.4) | 115 (26.4) | 87 (21.6) | 96 (23.4) | 111 (32.9) | 77 (23.8) | 40 (26.8) | 28 (26.4) | 827 (25.3) |
| No. (%) of calves born | 32 (12.6) | 40 (9.6) | 49 (11.4) | 45 (10.3) | 28 (6.9) | 21 (5.1) | 35 (10.4) | 28 (8.6) | 15 (10.1) | 8 (7.5) | 301 (9.2) |
| No. (%) of calves living at birth | 25 (9.9) | 34 (8.1) | 47 (11.0) | 33 (7.6) | 25 (6.2) | 19 (4.6) | 28 (8.3) | 27 (8.3) | 15 (10.1) | 8 (7.5) | 261 (8.0) |
| No. (%) of calves living 24 h | 23 (9.1) | 25 (6.0) | 38 (8.9) | 29 (6.7) | 21 (5.2) | 14 (3.4) | 24 (7.1) | 20 (6.2) | 13 (8.7) | 8 (7.5) | 215 (6.6) |
| No. (%) of calved slaughtered for research within 6 months after birth | 0 (0) | 2 (0.5) | 2 (0.5) | 3 (0.7) | 3 (0.7) | 0 (0) | 0 (0) | 1 (0.3) | 1 (0.7) | 0 (0) | 12 (0.4) |
| No. (%) of calves living at 6 months | 19 (7.5) | 19 (4.5) | 28 (6.5) | 17 (3.9) | 14 (3.5) | 9 (2.2) | 12 (3.6) | 13 (4.0) | 6 (4.0) | 3 (2.8) | 140 (4.3) |

†Somatic cell nuclear transfer. ‡Pregnancies were diagnosed by ultrasonography around 30 days of pregnancy. SCNT, somatic cell nuclear transfer.

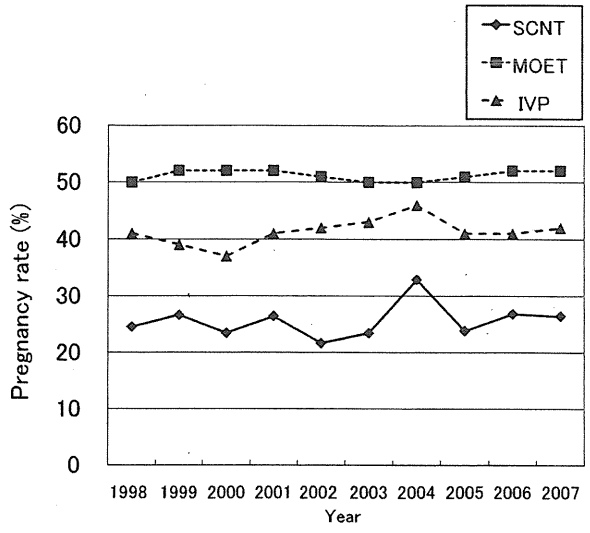


**Figure 1** Pregnancy rates of transferred bovine embryos derived from somatic cell nuclear transfer (SCNT), multiple ovulation and embryo transfer (MOET) and *in vitro* production of embryos (IVP) observed in Japan. The data for MOET and IVP are cited from statistics collected by the Ministry of Agriculture, Forestry and Fisheries of Japan (http://www.maff.go.jp/j/chikusan/sinko/lin/l_katiku/pdf/h19kekka.pdf).

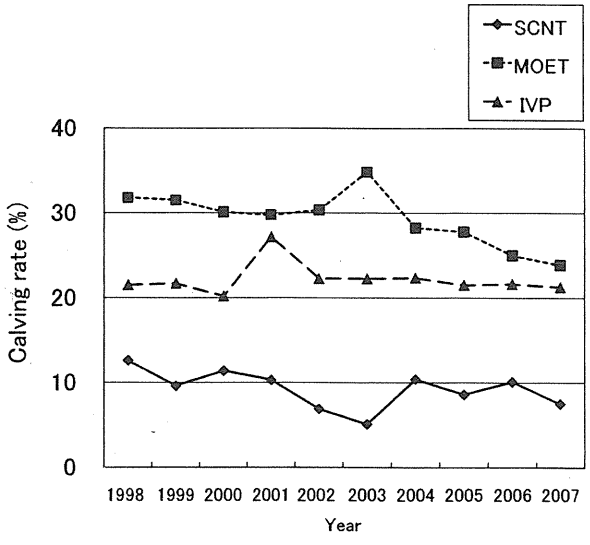


**Figure 2** Calving rates of transferred bovine embryos derived from somatic cell nuclear transfer (SCNT), multiple ovulation and embryo transfer (MOET) and *in vitro* production of embryos (IVP) observed in Japan. The data for MOET and IVP are cited from statistics collected by the Ministry of Agriculture, Forestry and Fisheries of Japan (http://www.maff.go.jp/j/chikusan/sinko/lin/l_katiku/pdf/h19kekka.pdf).

2002; Panarace *et al.* 2007), the abortions occurred spontaneously in recipient cows transferred with SCNT embryos (Table 2). It should be noted that 8.3–27.1% of the pregnant recipient cows lost their fetuses after 100 days of gestation. Such spontaneous abortions do not occur in MOET (Sartori *et al.* 2006) and IVP (Sakaguchi *et al.* 2002).

It has been considered that the survival of SCNT embryos depends on the reprogramming status of nuclear donor cells (Fairburn *et al.* 2002; Smith & Murphy 2004; Niemann *et al.* 2008; Oback 2009). To obtain appropriate reprogramming status of nuclear donor cells, studies concerning 'modification of the recipient oocyte', 'modification of the donor cells' and 'alternation in culture conditions for SCNT embryos' have been carried out (Campbell *et al.* 2007). In the case of Japan, such kinds of studies over the decade led the SCNT blastocyst formation rate to more than 35% (Kato & Tsunoda 2000; Kubota *et al.* 2000; Akagi *et al.* 2003, 2008). Although the use of selected donor cell lines for SCNT resulted in a 90% (9/10) calving rate (Urakawa *et al.* 2004), the present nationwide data (Table 1) suggest that SCNT embryos with unselected donor cell lines would not contribute toward improving the survival of SCNT embryos. In the research field of ET in cattle, a strategy concerning preparation of superior recipient cows with excellent feeding and reproductive management has been proposed for improving calf production efficiency (Peterson & Lee 2003; Looney *et al.* 2006). The strategy might also be effective in ETs with SCNT embryos.

## Survival of calves

The present survey revealed that transferred-embryo-based survival rates of calves derived from SCNT embryos were also not improved over the decade. The variations in survival rates for calves at birth, 24 h and 6 months after birth were 4.6–11.0%, 3.4–9.1% and 2.2–7.5%, respectively (Table 1). The survival level of SCNT calves in this survey was similar to those of other large-scale observations in cattle (Pace *et al.* 2002; Panarace *et al.* 2007). The calf loss observed in SCNT was due to high incidences of stillbirth, neonatal death and fatal diseases (Watanabe & Nagai 2009). To improve the survival of SCNT calves, care throughout the perinatal period from the viewpoints of nutrition and reproduction would be effective (FDA 2008). At the time of parturition, hormone treatment and/or operation of Caesarean section have been used frequently as effective moves to counter LOS (Kubota *et al.* 2000). With regard to newborn care, veterinary treatments such as artificial respiration, fluid replacement therapy and artificial feeding would improve survival rate and growth performance: a manual is available in Japanese for the types of care

**Table 2** Fate of pregnancies of recipient cows carrying embryos derived from somatic cell nuclear transfer over a decade (1998–2007)

| | | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. of pregnant recipients† | | 60 | 85 | 87 | 93 | 66 | 92 | 105 | 65 | 38 | 27 | 718 |
| No. (%) of abortions | <60 days | 13 (21.7) | 9 (10.6) | 12 (13.8) | 16 (17.2) | 17 (25.8) | 25 (27.2) | 16 (15.2) | 12 (18.5) | 4 (10.5) | 5 (18.5) | 129 (18.0) |
| | 61–99 days | 10 (16.7) | 13 (15.3) | 5 (5.7) | 20 (21.5) | 15 (22.7) | 28 (30.4) | 35 (33.3) | 12 (18.5) | 10 (26.3) | 7 (25.9) | 155 (21.6) |
| | >100 days | 5 (8.3) | 23 (27.1) | 21 (24.1) | 12 (12.9) | 6 (9.1) | 18 (19.6) | 19 (18.1) | 13 (20.0) | 9 (23.7) | 7 (25.9) | 133 (18.5) |
| No. (%) of recipients calved | | 32 (53.3) | 40 (47.1) | 49 (56.3) | 45 (48.4) | 28 (42.4) | 21 (22.8) | 35 (33.3) | 28 (43.1) | 15 (39.5) | 8 (29.6) | 301 (41.9) |

†Of all pregnant recipients, cows for which abortion times were unknown were excluded. Pregnancies were diagnosed by ultrasonography around 30 days of pregnancy.

listed here (http://nilgs.naro.affrc.go.jp/pub/report/report_No08.pdf).

## Effects of ovary storage on SCNT cattle production

After the occurrence of BSE in Japan (September 2001), all cattle in slaughterhouses were required to undergo BSE inspection. In addition, any materials originating from slaughtered cattle should be kept in slaughterhouses before inspection. Exceptions to this rule are that pelts and ovaries can be removed from slaughterhouses just after cattle are slaughtered; however, the use of these materials before inspection is forbidden by Japanese regulations. To plan countermeasures to the regulations, some Japanese researchers have carried out studies concerning the effect of storage of bovine ovaries at a low temperature, such as 10–15°C on the subsequent development of SCNT embryos reconstructed with the oocytes collected from the stored ovaries (Matsushita *et al.* 2004; Matsukawa *et al.* 2007). They concluded that such storage of ovaries for around 24 h did not affect blastocyst formation of resultant SCNT embryos in which the oocytes collected from the stored ovaries were used as recipient cytoplasts; however, the present study suggests negative effects of the ovary storage on SCNT cattle production.

When the decade (1998–2007) was divided into two periods, 'before BSE' (1998–2000) and 'after BSE' (2002–2007), no significant differences were observed between them in terms of the percentage of pregnant recipients; however, significant decreases were observed in the 'after BSE' period in terms of the percentages of calves born ($P < 0.01$), living calves at birth ($P < 0.05$), calves living 24 h ($P < 0.05$) and calves living 6 months ($P < 0.01$) (Table 3). With regard to abortions, the incidence during the first 61–99 days increased significantly ($P < 0.01$) in the 'after BSE' period (Table 4). These findings suggest the presence of negative effects on the quality of oocytes, which were used as recipients of SCNT, during the storage of ovaries at 10–15°C for 15–20 h. The negative effects might originate from the regeneration of cytoplasmic materials that fill oocytes.

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**Table 3** Survival of transferred bovine embryos and produced calves derived from somatic cell nuclear transfer (SCNT) in two periods divided by the occurrence of bovine spongiform encephalopathy (BSE) in 2001

| | Before BSE (1998–2000) | After BSE (2002–2007) | Total |
|---|---|---|---|
| No. of SCNT embryos transferred | 1099 | 1730 | 2829 |
| No. (%) of pregnant recipients† | 273 (24.8) | 439 (25.4) | 712 (25.2) |
| No. (%) of calves born | 212 (19.3) | 135 (7.8)** | 256 (9.0) |
| No. (%) of calves living at birth | 106 (9.6) | 122 (7.1)* | 228 (8.1) |
| No. (%) of calves living 24 h | 86 (7.8) | 100 (5.8)* | 186 (6.6) |
| No. (%) of calved slaughtered for research within 6 months after birth | 4 (0.4) | 5 (0.3) | 9 (0.3) |
| No. (%) of calves living at 6 months | 66 (6.0) | 57 (3.3)** | 123 (4.3) |

*$P < 0.05$, **$P < 0.01$ (by $\chi^2$ test). †Pregnancies were diagnosed by ultrasonography around 30 days of pregnancy.

**Table 4** Fate of pregnancies of recipient cows carrying embryos derived from somatic cell nuclear transfer (SCNT) in two periods divided by the occurrence of bovine spongiform encephalopathy (BSE) in 2001

| | | Before BSE (1998–2000) | After BSE (2002–2007) | Total |
|---|---|---|---|---|
| No. of pregnant recipients† | | 232 | 393 | 625 |
| No. (%) of abortions | <60 days | 34 (14.7) | 79 (20.1) | 113 (18.1) |
| | 61–99 days | 28 (12.1) | 107 (27.2)** | 135 (21.6) |
| | >100 days | 49 (21.1) | 72 (18.3) | 121 (19.4) |
| No. (%) of recipients calved | | 121 (52.2) | 135 (34.4)** | 256 (41.0) |

*$P < 0.05$, **$P < 0.01$ (by $\chi^2$ test). †Of all pregnant recipients, cows for which abortion times were unknown were excluded. Pregnancies were diagnosed by ultrasonography around 30 days of pregnancy.

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# WTPによる遺伝子組換え食品の社会的受容性の評価に係る研究

## Study on Evaluation of Social Acceptability of Genetically Modified Foods Based on WTP

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**Abstract.** This study intends to evaluate social acceptability of genetically modified (GM) foods from the two points of view "differences in acceptability by type of GM food (e.g. plant-based, animal-based, etc.)" and "differences in acceptability by GM characteristics (e.g. herbicide tolerance, high nutrient, etc.)". Surveys by web-based questionnaire led to the following conclusions. We recognized differences in consumer acceptability by food article. According to willingness to pay (WTP) for GM foods, consumer acceptability is lower than market valuation. And we also recognized differences in acceptability by GM characteristics.

**Key Words:** GM food, public acceptance, WTP, risk communication

## 1．背景と目的

遺伝子組換え食品（以下，GM 食品と表記）については，消費者の間で未だ「漠然とした不安」が残っているものと考えられる．そうした不安を解消していくためには，GM 食品に対する消費者の意識や受容性を把握し，適切なリスクコミュニケーションを継続的に展開していくことが重要である．また，一言に GM 食品と言っても，その原料となる遺伝子組換え体は多様であり，それを利用した食品も今後更に多様化していくことが予想される．GM 食品に対する受容性を把握する上では，そうした多様性を考慮した検討が必要である．

一括した食品群としてではなく，特定の食品を想定し，我が国の消費者の受容性を定量的に計測している研究として，寺脇（2003）[1]は，仮想市場評価法を用いて，「遺伝子組換えトウモロコシを原料として使用しているかどうかわからないコーン油」を「それを使用しないコーン油」に換えることに対する支払意思額（WTP: willingness to pay）を計測し，基準となる金額 600 円に対し，WTP の平均値が約 385 円，中央値が 250 円といった結果を示している．また，中村(2006)[2]は，組換えダイズ油，組換え豆腐，組換え餌の鮭，組換え鮭を対象に，GM 食品を避けることに対する WTP を計測し，組換え鮭の平均 WTP は組換えダイズ油より大きいなど，GM 食品の種類によって消費者の受容性が異なることを示している．

これらの研究からは，GM 食品を一括した食品群として扱うのではなく，具体的な情報を消費者に提示することの重要性が示唆されており，こうした具体的な食品を対象とした受容性の把握は更なる知見の蓄積が重要である．また，これまでの遺伝子組換え作物（以下，GM 作物と表記）は，害虫抵抗性や除草剤耐性といった生産者にメリットのある作物が主流であったが，今後，栄養成分改変や，環境耐性といった消費者や社会にとってメリットのある作物の開発が進むものと考えられる．こうした遺伝子組換えにより作物に与えられ

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る性質の違いは，消費者の受容性に影響を及ぼすものと推測される．

そこで，本研究では，次の 2 つの観点から GM 食品に対する消費者の受容性を把握することを目的とする．

① GM 食品の違いによる受容性の違い

「食品に用いられている組換え体が植物か，動物か，微生物か」，「原材料となる組換え体の原形が分かるか，分からないか」，「組換え体の直接的な摂取か，肥料や飼料としての利用による間接的な摂取か」といった GM 食品に係る諸要因の違いによる受容性の違いを把握する．

② GM 作物の世代の違いによる受容性の違い

遺伝子組換えにより作物に与えられる性質は様々である．ここでは，GM 作物をその性質の違いから次の 3 つの世代に分類し，GM 作物の世代の違いによる受容性の違いを把握する．

＜GM 作物の世代の分類と各世代の性質例＞
第 1 世代:除草剤耐性や害虫抵抗性等の性能を有し，主に生産者のメリットが期待される作物
第 2 世代:栄養成分改変によりビタミン等の含有量が増した，主に消費者のメリットが期待される作物
第 3 世代:耐乾燥等の環境耐性の性能を有し，社会的なメリットが期待される作物

## 2．研究方法

本研究では，一般消費者を対象とした次の 2 つの WEB アンケートを実施した．

### (1) GM 食品別受容性調査

ここでは，いくつかの GM 食品を想定し，非組換えの食品との比較による消費意向，及び GM 食品に対する WTP から消費者の受容性を把握する．なお，質問の順番による回答のバイアスを避けるため，調査画面上では，回答者ごとに食品の並びをランダムに表示させた．

表 1 GM 食品別受容性調査の実施要領

| 項目 | 内容 |
|---|---|
| 実施期間 | 2010 年 02 月 19 日～2010 年 02 月 22 日 |
| 有効回答 | 1,030 人<br>※性別年齢階層別の 10 セグメントに均等割付 |
| 調査項目 | 1) GM 食品別の消費意向<br>2) GM 食品別の WTP |

表 2 調査で想定した GM 食品

| 想定される要因 | | | 購買対象となる食品 | | |
|---|---|---|---|---|---|
| | | | 植物 | 動物 | 微生物 |
| 摂取方法 | 直接摂取 | 組換え体の原形が分かる | 組換えﾄｳﾓﾛｺｼを使った、ﾄｳﾓﾛｺｼの缶詰 | 組換えﾆﾜﾄﾘの鶏もも肉 | － |
| | | 組換え体の原形が分からない | 原料に組換えﾄｳﾓﾛｺｼを使ったｺｰﾝﾌﾚｰｸ | 組換えﾆﾜﾄﾘ由来成分を原料にした固形ｺﾝｿﾒ | － |
| | 間接摂取 | － | 肥料のｺｰﾝ油かすの原料に組換えｺｳﾓﾛｺｼを使ったﾄﾏﾄ | 組換えﾄｳﾓﾛｺｼを餌にして育ったﾆﾜﾄﾘの鶏もも肉 | 組換え微生物から抽出したｷﾓｼﾝを使って製造した、日本産ｶﾏﾝﾍﾞｰﾙﾁｰｽﾞ |

### (2) GM 作物の世代別受容性調査

ここでは，分類した各世代の GM 作物を原料とした GM 食品について，非組換え食品との比較による購入意向，及び GM 食品に対する WTP から消費者の受容性を把握する．

アンケートでは，回答者を 3 つのグループに分け，グループごとに異なる順番で第 1 世代～第 3 世代の各世代に係る質問を尋ねた．本稿では，各グループに対して最初に聞いた GM 作物の世代に対する回答結果（例えば，グループ 1 は第 1 世代の作物に対する回答）を示す．また，各世代の作物に係る質問をする際には，回答者にそれぞれの利点や懸念点に関する情報を提示し，その性質を理解してもらった上で回答を得た．

表 3 GM 作物の世代別受容性調査の実施要領

| 項目 | 内容 |
|---|---|
| 実施期間 | 2010 年 02 月 19 日～2010 年 02 月 22 日 |
| 有効回答 | 1,560 人　（520 人×3 グループ）<br>※性別年齢階層別の 10 セグメントに均等割付 |
| 調査項目 | 1) GM 作物の世代別の購入意向<br>2) GM 作物の世代別の WTP |
| 調査対象とした GM 作物の性質 | 【第 1 世代：除草剤耐性】<br>・除草剤をまいても枯れなくなるダイズ<br>・除草剤をまいても枯れなくなるトウモロコシ<br>【第 2 世代：栄養成分改変】<br>・オレイン酸を多く含んだダイズ<br>・ビタミンを多く含んだトウモロコシ<br>【第 3 世代：乾燥耐性】<br>・干ばつ・水不足に強いダイズ<br>・干ばつ・水不足に強いトウモロコシ |

## 3．結果

### (1) GM 食品別受容性調査

#### 1) GM 食品別の消費意向

「GM 食品が非組換えの食品よりも安い場合に，

食べてもよいと思うか」という質問に対して，食べてもよいとする回答者は約20～40%であり，約60～80%の人はGM食品を食べたくないと考えている．一方で，食品の種類によって食べてもよいとする割合は異なり，組換え体を間接的に摂取する食品の受容性は比較的高い結果となった．また「肥料のコーン油かすの原料に遺伝子組換えトウモロコシを使ったトマト」が45%と最も高く，一方「遺伝子組換えのニワトリの鶏もも肉」は22%と最も低かった．この2食品について$\chi^2$検定を行った結果，$\chi^2=120.4$ であり，2食品の消費意向には有意差がみられた．

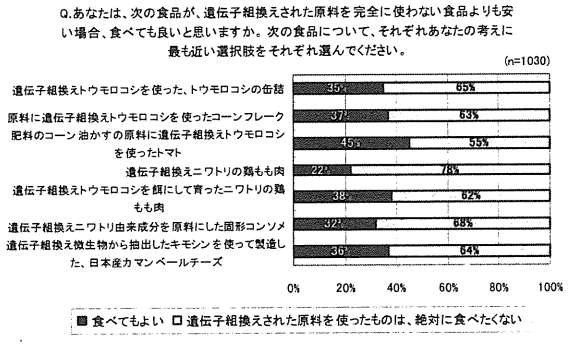


図1 GM食品の消費意向

2) GM食品別のWTP

1)で「非組換え食品よりも安ければ食べてもよい」とした約30%の回答者に対して，「遺伝子組換えされた原料を使っているかどうか分からない食品」に対するWTPを尋ねた．いずれのGM食品に対しても非組換え食品の設定価格より約30%低く，食品の違いによる差はみられなかった．

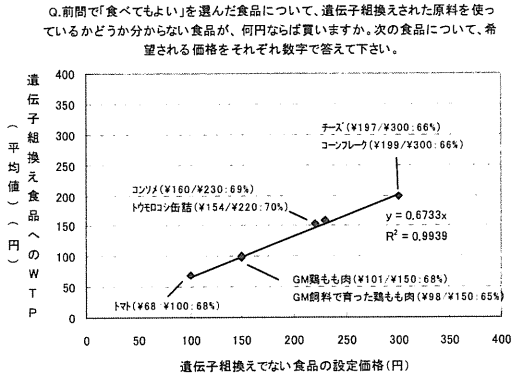


図2 非組換え食品の価格とGM食品のWTP

(2) GM作物の世代別受容性調査

1) GM作物の世代別の購入意向

「遺伝子組換えダイズを使った豆腐」，「遺伝子組換えトウモロコシを使ったトウモロコシの缶詰」を対象に，原料となるGM作物の世代ごとに，非組換え作物を原料とした食品と比較した際の購入意向を尋ねた．その結果，GM食品を買ってもよい（「同じ価格でもGM作物を使った食品を購入」，「安ければGM作物を使った食品を購入」，「どちらでもよい」）とした回答者と，GM食品は買わない（「高くても非組換え作物を使った食品を購入」）とした回答者の割合は，第1世代（除草剤耐性）では約30%と約60%，第2世代（栄養成分改変）と第3世代（乾燥耐性）では約50%と約40%であった．

また，第2世代については，「同じ価格でもGM作物を使った食品を購入する」とした回答者が約10%おり，他の2世代と比べて，積極的に購入するとした回答者が多かった．

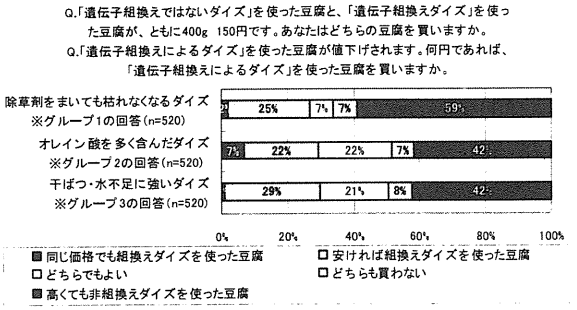


図3 組換えダイズの豆腐の購入意向

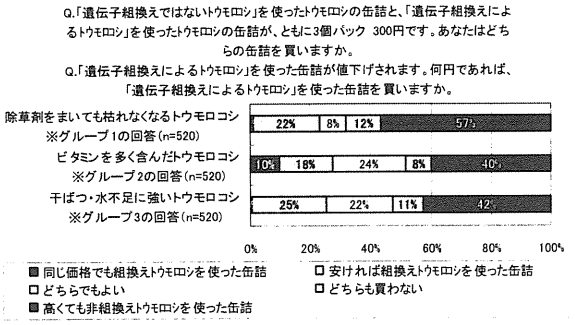


図4 組換えトウモロコシの缶詰の購入意向

2) GM作物の世代別のWTP

「安ければGM作物を使った食品を購入する」とした回答者に対して，GM作物を原料とした食品に対するWTPを尋ねた．組換えダイズを使った豆腐，組換えトウモロコシを使ったトウモロコシの缶詰共に，第2世代，第3世代，第1世代の順にWTPが低下する結果となった．また，各WTP

を対数変換しt検定を行った結果，組換えダイズを使った豆腐において，第1世代と第2世代の間に有意水準5%の有意差がみられた．

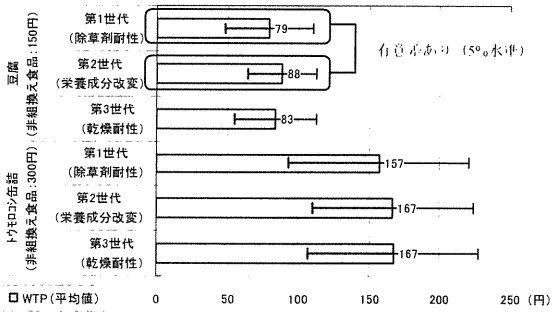


図5 GM作物の世代別のWTPの比較

## 4．考察

### (1) GM食品の違いによる受容性の違い

約70%の人が「非組換え食品より安い場合でもGM食品を食べたくない」と回答しており，GM食品を食べることに対する消費者の抵抗感の大きさが伺える．ただし，食品の違いにより消費意向に違いがみられ，間接摂取の場合は受容性が比較的高く，一方，動物の組換え体については低い傾向がみられた．既存研究と同様に，GM食品を一括の食品群として扱うのではなく，個々の組換え体，食品の違いを考慮したコミュニケーションの重要性が示された．

「非組換え食品より安ければ食べてもよい」とした約30%の人が回答した「遺伝子組換えされた原料を使っているかどうか分からない食品」に対するWTPからは，食品の違いによる差はみられなかった．これは，「安ければ食べてもよい」とする一定の受容性を有するサンプルのみの回答であることなどが影響しているものと推察される．

また，WTPはいずれの食品に対しても非組換え食品の設定価格より約30%低かったが，GM作物に関する先物市場の動向をみると，東京穀物商品取引所での「一般大豆先物」の「Non-GMO大豆先物」に対する価格比率は，2011年2月限価格（2010年2月末時点）で約16%低い．GM作物に対しては，市場の評価額と消費者の意識の間に乖離があり，消費者の受容性は市場の評価よりも低いものといえる．

### (2) GM作物の世代の違いによる受容性の違い

現状のGM作物の主流である第1世代の作物よりも，第2世代，第3世代の作物の購買意向が強く，第2世代，第3世代に対しては，GM食品を買ってもよいとする回答が半数を超えた．GM作物の性質について消費者に情報を伝えていくことで，第2世代や第3世代の作物については，従来よりも円滑にコミュニケーションが進む可能性が伺える．

また，第2世代に対しては積極的に購入するとした回答が約10%おり，また，WTPについても第1世代との間に有意差がみられた．組換えのメリットが消費者に享受される性質であることから，魅力が高いと判断されたものと考えられる．一方，社会的な観点からのメリットが大きい第3世代に対しては，積極的に購入するといった回答は少なく，より踏み込んだ情報提供やコミュニケーションが重要であると考えられる．

## 5．結論

GM食品に対する消費者の受容性は，食品の違いや，GM作物が有する性質の違いにより異なることが示された．また，WTPを把握することにより，評価対象とする食品間の受容性の違いや，市場の動向と消費者意識の乖離の程度を定量的に把握することができた．GM食品に関するコミュニケーションにおいては，こうした受容性の違いを踏まえ，消費者に提供する情報の具体化や重点化を検討していく必要がある．また，本研究では，こうした受容性の違いが消費者のどのような意識や属性に由来するものなのかは十分に把握できておらず，今後，質的な調査等による更なる消費者意識の解明が求められる．

謝辞

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# Novel Method to Detect a Construct-Specific Sequence of the Acetolactate Synthase Gene in Genetically-Modified Flax CDC Triffid (FP967)

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**During the fall of 2009, a trace of unauthorized genetically modified (GM) flax (*Linum usitatissimum* L.) line, CDC Triffid, which is resistant to sulfonylurea herbicides, was detected in many countries including Japan. A method to reliably identify the CDC Triffid line was urgently required. We developed a novel construct-specific real-time polymerase chain reaction (PCR) method to identify the mutant acetolactate synthase gene in the CDC Triffid line. We confirmed that the method can detect 0.001% GM flax in DNA mixing solution. The study shows that the developed method is specific, sensitive and reliable way to monitor a trace of CDC Triffid.**

**Key words** genetically modified organism; flax; polymerase chain reaction; genomic DNA

*Linum usitatissimum* L., commonly known as flax, is an important oilseed crop that provides diversity in crop rotations in Canada. Because some sulfonylurea herbicides are very persistent in soil, the choice of crops for use in rotations is limited.[1)] To address this problem, McHughen developed a sulfonylurea-resistant flax line by introducing acetolactate synthase (ALS) gene cloned from *Arabidopsis thaliana* using *Agrobacterium*-based transformation.[1—3)] The 5.8-kb ALS gene construct contains the ALS gene and its flanking regions. The coding sequence of the gene is modified by a single cytosine-to-thymine substitution at the 589th nucleotide, which results in reduced affinity for sulfonylurea herbicides.[1)] As well as the ALS gene, the construct also contains a neomycin phosphotransferase II gene (*nptII*), a spectinomycin/streptomycin resistance gene (Spec), a $\beta$-lactamase gene (*bla*), and the nopaline synthase gene (NOS). Expression of the resulting sulfonylurea herbicides resistance trait was subsequently demonstrated in progeny, and the transgenic line was designated CDC Triffid (FP967).[4)]

After safety assessment by the Canadian Government, the CDC Triffid line was authorized for food and feed use in Canada. It was also approved for food and feed use in the United States in 1998. However, this line has not been approved for use in food and feed in any other countries.

On September 8, 2009, Germany posted a notification on an internal EU system (Rapid Alert System for Food and Feed-RASFF) that a trace of the unauthorized genetically modified (GM) line CDC Triffid was detected in a Canadian shipment, following reports from other European countries. Japan and the EU have a policy of zero tolerance for unauthorized GM crops[5,6)]; therefore, a method to specifically detect the CDC Triffid line, the only cultivated GM flax, was urgently required. The Joint Research Centre (JRC) of the EU detected the CDC Triffid line using a construct-specific method, which was based on detection of the region between NOS terminator (NOST) and Spec in CDC Triffid (http://gmo-crl.jrc.ec.europa.eu/flax.htm). However, an additional method is required to unequivocally confirm the CDC Triffid line, because other unauthorized GM crops may contain constructs with similar NOST-Spec regions. In the present study, we developed a specific, sensitive and reliable method based on the ALS gene of *A. thaliana* to qualitatively detect CDC Triffid.

## MATERIALS AND METHODS

**Flaxseed Materials** Non-GM flaxseed (Senbirishu; JP No. 31750, Stock No. 35490) was purchased from the NIAS Genebank of Japan. Flax grains (Sample 4) imported from Canada, which were suspected to be contaminated with CDC Triffid based on testing results, were purchased online. Seeds of CDC Triffid were kindly provided from Mr. Michael Scheffel, National Manager of Seed Section in Canadian Food Inspection Agency.

**Purification of DNA** Flaxseeds were ground using a mixing mill. DNA was extracted and purified from a portion (0.5 g) of the ground sample, using an ion-exchange resin-type DNA extraction and purification kit (Genomic-tip; QIAGEN, Hilden, Germany) as follows: G2 buffer (7.5 ml) and $\alpha$-amylase (20 $\mu$l) were added to the sample and mixed thoroughly with a vortex mixer. The mixture was incubated at 37 °C for 1 h before addition of 7.5 ml G2 buffer, 200 $\mu$l Proteinase K, and 20 $\mu$l RNase A. The mixture was vortexed and incubated at 50 °C for 1 h. During incubation, the samples were mixed several times by inverting the tubes. The samples were then centrifuged at 5000×***g*** at 4 °C for 15 min. The supernatant was divided into 2 ml aliquots and centrifuged in polypropylene centrifuge tubes at 20000×***g*** at 4 °C for 15 min. The supernatant was applied to a Genomic-tip 20/G column (QIAGEN) pre-equilibrated with 1 ml QBT buffer. The tip was washed three times with 2 ml QC buffer and transferred to a fresh centrifuge tube, and 1 ml pre-warmed QF buffer (50 °C) was added to elute DNA. The DNA sample was transferred to a centrifuge tube, an equal volume of isopropyl alcohol was added, and the sample was mixed thoroughly. DNA was collected by centrifugation at 12000×***g*** for 15 min. The pellet was rinsed with 1 ml 70% (v/v) ethanol and centrifuged at 12000×***g*** for 3 min. The supernatant was discarded and the precipitate was dried with an aspirator. The DNA was dissolved in 50 $\mu$l water for use in analyses. The DNA was quantified by measuring UV absorption at 260 nm using an ND-1000 spectrophotometer (NanoDrop Technologies Inc., DE, U.S.A.).

**Polymerase Chain Reaction (PCR)** Each PCR reaction mixture (25 $\mu$l) contained 2.5 $\mu$l 10×PCR buffer II (Applied Biosystems, CA, U.S.A.), 0.16 mM dNTP, (Applied Biosystems), 1.5 mmol/l $MgCl_2$, 1.2 $\mu$mol/l 5′ and 3′ primers, 0.8 U AmpliTaq Gold (Applied Biosystems) and 10 ng template

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DNA. The PCR conditions were as follows: 95 °C for 10 min, followed by 50 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 30 s, extension at 72 °C for 60 s, and terminal elongation at 72 °C for 7 min. PCRs were carried out using the GeneAmp PCR System 9700 (Applied Biosystems).

To determine the nucleotide sequence of the transgenic construct harbored in the CDC Triffid line, DNA fragments were amplified by PCR using the following primer set: als3F primer, 5′-CAA GCC TTA ACC CGC TCT TC-3′, and als3R primer, 5′-TCG TAA TCG AAC GCG TTA CC-3′. After PCR, amplified products were analyzed by electrophoresis on agarose gels.

**DNA Sequencing** PCR-amplified DNA fragments were extracted from agarose gels and purified using a QIAquick PCR purification kit (QIAGEN). DNA fragments were directly sequenced from both strands using forward and reverse primers with an ABI PRISM 3700 DNA analyzer and the Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions. Nucleotide sequences were analyzed using Lasergene ver. 7.0 software (DNASTAR Inc., WI, U.S.A.).

**Real-Time PCR** Real-time PCR assays were performed using an ABI PRISM™ 7900 Sequence Detection System (Applied Biosystems). The 25 $\mu$l reaction mixture consisted of 2.5 $\mu$l sample DNA solution (25 ng), 12.5 $\mu$l Universal Master Mix® (Applied Biosystems), 0.8 $\mu$M each primer, and 0.1 $\mu$M probe. The PCR conditions were as follows: 2 min at 50 °C, 95 °C for 10 min followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The *A. thaliana*-derived ALS gene with the single substitution mutation was detected using the following primers: AtALSc870t MGB F, 5′-TGC GTT GTT AGA TAG TGT TCC TCT TG-3′, and AtALSc870t MGB R, 5′-CGC ATC TGT ACC AAT CAT ACG AC-3′, and the probe sequence was AtALSc870t MGB, 5′-FAM-CAG GAC AAG TCT CTC-MGB-3′. The primers and probe used to detect the NOST-Spec region of CDC Triffid were designed on the basis of information provided by JRC (http://ec.europa.eu/dgs/jrc/index.cfm). The primers were as follows: NOST-Spec F, 5′-AGC GCG CAA ACT AGG ATA AA-3′; NOST-Spec R, 5′-ACC TTC CGG CTC GAT GTC TA-3′; and the NOST-Spec probe sequence was 5′-FAM-CGC GCG CGG TGT CAT CTA TG-BHQ1-3′. The primers and probe used to detect the flax internal control gene, stearoyl-ACP desaturase II (SAD; GenBank no.: X70962.1), were as follows: SAD F, 5′-GCT CAA CCC AGT CAC CAC CT-3′; SAD R, 5′-TGC GAG GAG ATC TGG AGG AG-3′; SAD probe, 5′-FAM-TGT TGA GGG AGC GTG TTG AAG GGA-BHQ1-3′. All primers and probes were diluted with an appropriate volume of distilled water, and stored at −20 °C until use. Results were analyzed using SDS 2.1 sequence detection software on the ABI PRISM™ 7900 Sequence Detection System (Applied Biosystems).

**Real-Time PCR Data Analysis** Typically, the baseline was set to cycles 3 through 15. The ΔRn threshold for plotting Ct values was set to 0.2—0.5 during exponential amplification. Reactions with a Ct value of less than 43 and exponential amplification plots were scored as positive. If a Ct value could not be obtained, the reaction was scored as negative. Reactions with a Ct value of less than 43, but without exponential amplification as judged by visual inspection of the respective ΔRn plots and multicomponent plots were scored as negative.

## RESULTS AND DISCUSSION

**Identification of the Mutated ALS Gene in Flax DNA** To detect the ALS sequence derived from *A. thaliana* (GenBank no: AY124092.1) in samples of flaxseeds, we used genomic DNAs purified from CDC Triffid seed and Sample 4 as templates for the PCR reaction (Fig. 1). The primers were designed to amplify the mutant ALS containing a single nucleotide substitution that results in a proline-to-serine substitution in the mature enzyme. This amino acid substitution results in reduced affinity for chlorsulfuron.[3] Electrophoresis of the PCR products showed that a single 277 bp band was specifically detected in CDC Triffid, whereas the band was not detected in non-GM (Senbirishu) samples (Fig. 1A). Direct sequence analysis of the amplified product showed the position of the specific mutation in the inserted ALS sequence, which was originally derived from *A. thaliana* (Fig.

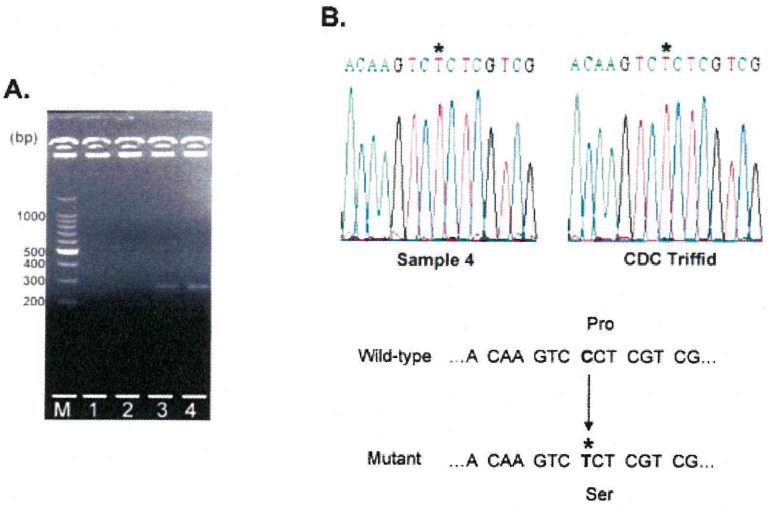


Fig. 1. Identification of Mutant ALS Gene in CDC Triffid

(A) Agarose gel electrophoresis of the PCR-amplified product using als3F/R primers. M: 100-bp DNA ladder marker, 1; non-template control, 2; non-GM flax (Senbirishu), 3; Flax sample contaminated with CDC Triffid (Sample 4), 4; CDC Triffid line as template (B) Sequence of PCR product. Asterisk indicates position of mutation.

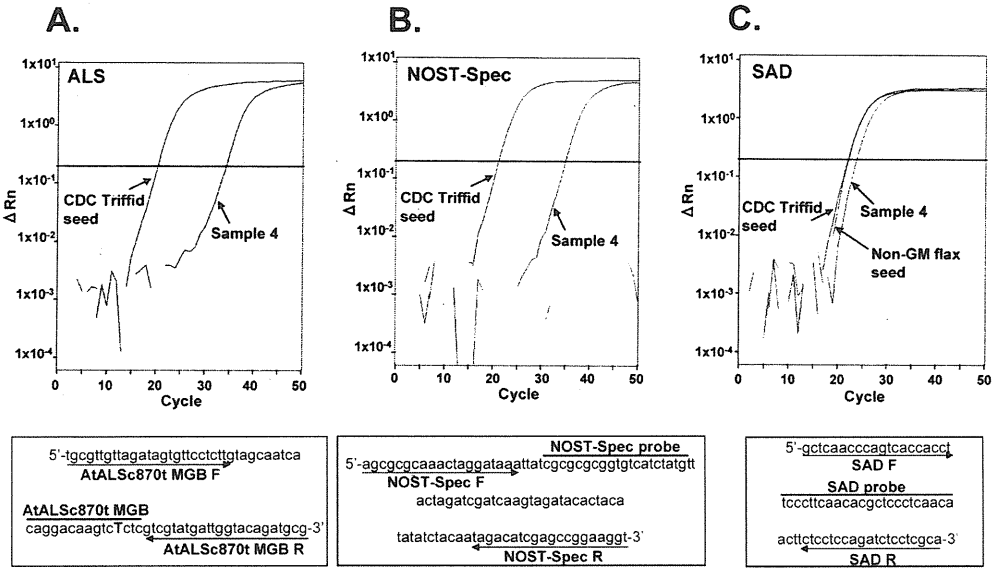


Fig. 2. Amplification Plots of Real-Time PCR Analysis for Construct-Specific Detection of CDC Triffid

Real-time PCR analyses were performed using primers and probes designed from mutant ALS in CDC Triffid (A), from NOST-Spec construct (B), and from internal control, SAD (C). PCR amplifications were conducted using 25 ng genomic DNA purified from each product as template. Ct values of non-GM flaxseed, CDC Triffid seed, and Sample 4 were negative, 20.34 and 34.26, respectively, in mutant ALS gene analysis (A); negative, 21.20 and 34.97, respectively, in NOST-Spec construct analysis (B); and 22.06, 21.98 and 23.74, respectively, in SAD gene analysis (C). Threshold value was set at 0.20. Sequences of amplicons for A, B and C are shown under their respective amplification curves.

1B). Sample 4 showed an identical band to that of CDC Triffid seed, confirming that this grain batch is contaminated with transgenic material.

**Construct-Specific Identification of the CDC Triffid ALS Gene** We designed primer-probe sets to detect the recombinant ALS gene sequence in a real-time PCR assay. The primers/probes were designed on the basis of the partial sequence described above (sequences of primers/probes are given in Materils and Methods; amplicon sequence is shown in Fig. 2A). Two primer–probe sets were used to identify the construct-specific CDC Triffid line in the real-time PCR assay. One primer-probe set detects the recombinant ALS sequence containing a single nucleotide substitution in the CDC Triffid construct (Fig. 2A). The other set, which was designed by JRC, detects the NOST-Spec region as the specific target for CDC Triffid. Figure 2 shows amplification plots with both primer-probe sets using genomic DNA from non-GM flax, CDC Triffid seed, and Sample 4. The amplicons derived from CDC Triffid seed and the Sample 4 consisted of 73 bp of ALS (Fig. 2A) and 105 bp of NOST-Spec (Fig. 2B), whereas those target sequences were not detected in the non-GM flax. The internal control, SAD (target size, 68 bp), was detected in all samples (Fig. 2C).[7] None of the primer-probe sets showed non-specific amplification using template genomic DNA derived from non-GM maize, rice, soybean or canola (data not shown). The limit of detection (LOD) in the real-time PCR methods were examined by diluting template genomic DNA purified from CDC Triffid with that of non-GM flax. The LODs of the both methods using ALS and NOST-spec primer-probe sets were found to be 0.001% GM flax in 25 ng DNA mixing solution (data not shown).

In the present study, we identified the region in the mutated ALS sequence that was originally derived from *A. thaliana* in CDC Triffid seed, and developed a specific and sensitive real-time PCR detection method targeting this sequence. The method is a simple and reliable assay for monitoring a trace of CDC Triffid, as detected in Sample 4. Further studies are required to determine the LOD of CDC Triffid in flax sample quantitatively, and to determine whether the method can also be used to detect CDC Triffid in processed food or feed containing flax or flax derivatives.

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# Identification and Detection Method for Genetically Modified Papaya Resistant to Papaya Ringspot Virus YK Strain

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**Unauthorized genetically modified (GM) papaya (*Carica papaya* LINNAEUS) was detected in a commercially processed product, which included papaya as a major ingredient, in Japan. We identified the transgenic vector construct generated based on resistance to infection with the papaya ringspot virus (PRSV) YK strain. A specific detection method to qualitatively monitor papaya products for contamination with the GM papaya was developed using the real-time polymerase chain reaction.**

**Key words** genetically modified organism; papaya; polymerase chain reaction; genomic DNA

Papaya (*Carica papaya* LINNAEUS) is an important fruit crop in tropical and subtropical areas.[1] Infection with the papaya ringspot virus (PRSV) causes disastrous damage to papaya harvests.[2] In response to this problem, genetically modified (GM) papayas have been developed in various places, such as Hawaii, Florida, China, Jamaica, Taiwan, Thailand, Australia, Malaysia, Philippines and Vietnam.[2]

Japan has announced a mandatory safety assessment of GM foods and processed foods containing GM ingredients, and the importation of any unauthorized GM foods to Japan has been prohibited since April 1, 2001. Therefore, the establishment of qualitative detection methods for unauthorized GM foods was required for monitoring purposes. We previously developed and reported qualitative detection methods for various GM crops, such as potato,[3,4] maize,[5–8] rice,[9] and flax,[10] using polymerase chain reaction (PCR) methods. In the case of papaya, we established a qualitative detection method for GM papaya (Line 55-1), which was the first commercialized PRSV-resistant GM papaya developed in Hawaii, using a PCR test and a histochemical assay.[11–13] A safety assessment for Line 55-1 by the Food Safety Commission of Japan was finished in 2009.[14] Since Japan imports many papayas from Southeast Asia, we are required, in Japan, to monitor commercially processed products that include papaya as a major ingredient for contamination with other unauthorized GM papayas generated in the region.

GM papayas carry the transgenic vector construct generated based on resistance to PRSV infection by expressing the PRSV's coat protein (CP) gene. Since the other unauthorized GM papayas developed may differ in the transgenic vector construct of the authorized GM papaya (Line 55-1), we developed a method for detecting contamination with unauthorized GM papaya. In the present study, we found the unauthorized GM papaya, PRSV-YK, in processed products containing papaya as a major ingredient, papaya-leaf-tea, pickles and jam, and developed a method for the detection of PRSV-YK using the real-time PCR.

## MATERIALS AND METHODS

**Papaya Samples** Papaya products were purchased through the internet in Japan. Hawaiian non-GM papaya (Sunset) fruit was purchased from a Japanese trade agency *via* the Hawaii Papaya Industry Association through the Consumer Affairs Agency, Government of Japan.

**Purification of DNA** Dried papaya leaves in papaya-leaf-tea, papayas in pickles and Sunset sarcocarp were ground using a mixing mill. Papaya jam was used for purification of DNA without grinding. DNA was extracted and purified from 2 g of the samples using an ion-exchange resin-type DNA extraction and purification kit (Genomic-tip; QIAGEN, Hilden, Germany) as follows: 30 ml Buffer G2 (QIAGEN), 20 $\mu$l 100 mg/ml RNase (QIAGEN) and 500 $\mu$l cellulase (Sigma-Aldrich, St. Louis, MO, U.S.A.) were added to the sample and vortexed thoroughly, then incubated at 50 °C for 1 h. The mixture was incubated at 50 °C for another 1 h after the addition of 200 $\mu$l Proteinase K (QIAGEN). During the incubation, the samples were mixed several times by inverting the tubes. The samples were then centrifuged at 3000×***g*** at 4 °C for 20 min. The supernatant was applied to a Genomic-tip 100/G column (QIAGEN), which was pre-equilibrated with 4 ml Buffer QBT (QIAGEN). The tip was washed three times with 7.5 ml Buffer QC (QIAGEN) and transferred to a fresh centrifuge tube, and 3 ml pre-warmed Buffer QF (QIAGEN) (50 °C) was added to elute the DNA. The DNA sample was transferred to a centrifuge tube, an equal volume of isopropyl alcohol was added, and the sample was mixed thoroughly. DNA was collected by centrifugation at 12000×***g*** for 15 min. The pellet was rinsed with 1 ml 70% (v/v) ethanol and centrifuged at 12000×***g*** for 3 min. The supernatant was discarded and the precipitate was dried. The DNA was dissolved in 20 $\mu$l water for use in analyses. The DNA was quantified by measuring UV absorption at 260 nm using a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, U.S.A.).

**PCR** Each PCR reaction mixture (25 $\mu$l) contained 2.5 $\mu$l 10×PCR buffer II (Life Technologies, Carlsbad, CA, U.S.A.), 0.16 mM of each deoxyribonucleotide triphosphate (dNTP) (Life Technologies), 1.5 mM $MgCl_2$, 1.2 $\mu$M forward and reverse primers, 0.8 U AmpliTaq Gold (Life Technologies) and 25 ng template DNA. The PCR conditions were as follows: 95 °C for 10 min, followed by 50 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 30 s, extension at 72 °C for 60 s and terminal elongation at 72 °C for 7 min. PCRs were carried out using the GeneAmp PCR System 9700 (Life Technologies). To determine the nucleotide sequence of the transgenic vector construct harbored in GM

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