Genetic Transformation of Egg Shell Color in Chicken by the Use of Irradiated Sperm

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Genetic transformation of egg shell color has been attained in chickens by the use of gamma ray irradiated sperm. Double artificial inseminations were used. Recipient females were inseminated with irradiated semen from a donor strain with brown eggs and then 24 hours later with normal unirradiated semen from the recipient strain with white eggs. The frequency of transformation of egg shell color genes was 3.8% in the first progeny generation. Transferred genes were stable for at least four successive generations, and were integrated from generation to generation under selective mating. This technique may be useful for improving qualitative characters of the chicken strain in a relatively short time.

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Key words : chicken, genetic transformation, gamma-ray irradiated sperm, egg shell color, pseudofertilization

Introduction

There are many reports on the production of transgenic mammals by microinjection of foreign DNA into the male pronucleus of fertilized ova (BRINSTER *et al.* 1985, HAMMER *et al.* 1985, WAGNER *et al.* 1981). Other techniques such as the use of retroviral vector (RUBENSTEIN *et al.* 1986) and electroporation (POTTER *et al.* 1984) are also used. To date reports on transgenic poultry are scanty, despite the feasibility and potential economic importance of the technology (CRITTENDEN and SALTER 1985, FREEMAN and MESSER 1985, WAGNER 1985). Attempts to alter the chicken germline by gene transfer into follicles or *in vitro* cultured early embryos have not been successful (SHUMAN and SHOFFNER 1986). In order to produce transgenic chickens a novel method of gene transfer should be developed.

PANDEY and PATCHELL (1982) have attained genetic transformation of egg shell and feather color in chickens by the use of heavily γ -ray irradiated sperm. They showed that genes coding for egg shell or feather color could be transferred in a frequency of 3.5% to the progeny through irradiated spermatozoa. The present studies have been conducted to verify the "genetic transformation technique" as described by them in order to develop a highly productive brown egg strain.

Materials and Methods

Two strains of chickens were used. A White Leghorn G strain (WL-G) which has

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white feathers, pure white eggs and high egg production was used as the recipient. A strain of Rhode Island Red Y 8 birds (RIR-Y 8) which has brown feathers and brown eggs was served as donors.

Semen was collected from RIR-Y 8 and WL-G males by abdominal massage (BOGDONOFF and SHAFFNER 1954). Semen samples were diluted three-fold with Ringer solution.

Sperm motility, Sperm survival rate and semen pH were examined. Sperm motility and survival rate were estimated microscopically by eye. Sperm motility score was indicated as follows : very high (+++), high (++), medium (+) and low ($\pm$). Sperm survival rate was given as the ratio of the number of dead sperm over the total sperm counts. Semen pH was checked by pH paper.

Pooled semen collected from the RIR-Y 8 males was divided into two parts. One part was kept at room temperature while the other part was irradiated by γ -ray of ^{60}Co source (Cobalt 60 Gamma Ray Irradiation System, Faculty of Engineering, Nagoya University). Irradiation was done at 370, 770 and 1160 Gy at room temperature for 20 to 60 minutes at a distance 10 ~ 15 cm from the source of irradiation. WL-G females were first inseminated with the irradiated semen. The next day (about 24 hours later), they were further inseminated with freshly collected unirradiated WL-G semen.

The dose of inseminations was 0.2 ml per female for irradiated or unirradiated semen. Two days after insemination, eggs laid were collected for two weeks and kept at 12°C until incubation.

Incubation was carried out at 37.8°C for 21 days under relative humidity of 60 ~ 80%. Newly hatched chicks were checked for sex and plumage color. All chickens were fed a standard commercial layer diet (Chick-food for up to 0 ~ 2 weeks, Chusu for 2 ~ 6 weeks, Daisu for 6 weeks ~ maturity and L-mash after maturity, each diet is shown after registered name of manufacturer, Nihon Nosan Kogyo and Co., Ltd. and water was given *ad libitum*.

Photometric measurements of egg shell color were carried out with a reflection photo difference meter (Tokyo Denshoku & Co., Ltd, TC-1500 MC) (brightness : L value, red component : a value and yellow component : b value) at the blunt end of the egg. These three values of strains and generations were examined statistically. For the synthetic estimates of egg shell color, Z values were calculated for each egg by a discriminate function formula using three color values. The formula used was $Z = a_1(x_1 - \bar{x}_1) + a_2(x_2 - \bar{x}_2) + a_3(x_3 - \bar{x}_3)$, where $a_1 = 0.0429053$, $a_2 = 0.162609$, $a_3 = 0.166231$, $x_1 = \text{L value}$, $x_2 = \text{a value}$ and $x_3 = \text{b value}$, respectively.

Each generation from the second to the fourth of genetic transformation chickens was produced as follows : the second generation was produced by mating of random selected males and selected females laid brownish colored eggs. The third generation was produced by mating of selected males whose mother hens laid the colored eggs and the selected females laid the colored eggs. The fourth generation was produced exactly by the same way as that of the third generation.

Results

Irradiation adversely affected sperm survival rates with levels of irradiation (Table 1). Motility of surviving sperm was slightly reduced after irradiation at all doses of irradiation. Semen pH increased in all irradiation groups (Table 1).

When the WL-G hens were inseminated with only the irradiated semen of RIR-Y 8, no fertilized eggs were obtained regardless of the intensity of irradiation.

In an attempt to make transgenic chickens, we decided to use the semen irradiated by 770 Gy. The first and subsequent generations of the transgenic chickens were designated as WL-r 1, WL-r 2, WL-r 3, etc.

The 77 WL-G females laid a total of 956 eggs in two weeks. The number of chicks (WL-r 1) hatched was 714 (hatchability 75%, 378 males, 336 females). There were no differences in the rate of malformation and feathering between WL-r 1 and control WL-G chicks. Among the WL-r 1 female chicks, 312 out of 336 survived until 180 days of age. Of the 312 WL-r 1 females which started laying, 12 (3.8%) laid brownish colored shell eggs.

Egg production performances (egg weight at 240 days of age, body weight at 240 days of age and egg laying rate during 151 ~ 270 days of age) of WL-r 1 females were almost the same as those of the original WL-G females (Table 2).

The genetic transmittance of the colored egg shell gene(s) was examined by successive selective matings to the females with brownish egg shell color. Number of

Table 1. Effects of γ -ray irradiation on sperm motility, sperm survival rate and semen pH of RIR-Y 8

Irradiation dose (Gy)	Sperm motility	Sperm survival rate (%)	Semen pH
0	+ ^a	80~90	6.6~6.8
370	+	60~70	7.2
770	+	30~40	7.6
1160	$\pm$	0	7.2

^a: "+" and " $\pm$ " indicate medium and low motility, respectively.

Table 2. Egg production performances of WL-r 1 and WL-G

Strain	No. of females	Egg weight at 240 days (g)	Body weight at 240 days (g)	Egg production rate at 151~270 days (%)
WL-r 1	26	56.8 $\pm$ 4.0 ^a	1635 $\pm$ 151	89.4 $\pm$ 8.7
WL-G	288	56.4 $\pm$ 4.2	1614 $\pm$ 147	90.6 $\pm$ 9.5
WL-G ^b		56.8	1681	89.7

^a: Mean $\pm$ S.D.

^b: Average of 4 years

females laid colored shell eggs was 12/312 (3.8%) in WL-r 1, 8/297 (2.7%) in WL-r 2, 8/74 (10.8%) in WL-r 3, and 19/62 (30.6%) in WL-r 4, respectively.

Photometric variations of egg shell color (L value, a value and b value) in RIR-Y 8, WL-G and their hybrids were measured (Table 3). Brightness (L value) of RIR-Y 8 eggs were remarkably low as compared with that of WL-G eggs. Red (a value) and yellow (b value) components in RIR-Y 8 were high compared with those in WL-G. Three color values between strains were significant ($P < 0.01$). Table 4 shows photometric values of egg shell color from WL-r 1 to WL-r 4. L value of WL-r 4 was almost seven points lower than that of WL-r 1. The a value was negative in all generations. The b value was increased in WL-r 4 compared with that in WL-r 1. All three color values of WL-r 4 showed the best points among the successive four generations, but still do not reach to the parental strain RIR-Y 8 and (RIR-Y 8 $\times$ WL-G) F₁. Egg shell color values between WL-r 1 and WL-r 4 were statistically

Table 3. Photometric values of egg shell color of RIR-Y 8, WL-G and F₁

Strain	No. of eggs	L-value (brightness)	a-value (red component)	b-value (yellow component)
RIR-Y 8	147	61.48 $\pm$ 3.30 ^{*,a}	5.26 $\pm$ 0.78 ^a	18.94 $\pm$ 1.10 ^a
(RIR-Y 8 $\times$ WL-G) F ₁	243	76.60 $\pm$ 4.00 ^b	1.59 $\pm$ 1.61 ^b	16.10 $\pm$ 2.19 ^b
WL-G	549	87.88 $\pm$ 1.04 ^c	-1.56 $\pm$ 0.30 ^c	5.23 $\pm$ 1.83 ^c

*: Mean $\pm$ S.D.

^{a,b,c}: Values with different superscripts differ significantly at $P < 0.01$.

Table 4. Photometric values of egg shell color of transgenic chickens in successive generations (WL-r 1, r 2, r 3 and r 4)

Generation	No. of eggs	L-value (brightness)	a-value (red component)	b-value (yellow component)
WL-r 1	60	85.65 $\pm$ 5.47 ^{*,a}	-2.15 $\pm$ 0.51 ^a	12.26 $\pm$ 1.33 ^a
WL-r 2	40	82.77 $\pm$ 3.46	-1.91 $\pm$ 0.61 ^b	12.20 $\pm$ 1.77
WL-r 3	40	82.85 $\pm$ 0.76	-1.68 $\pm$ 0.24 ^c	10.80 $\pm$ 0.91 ^b
WL-r 4	95	78.75 $\pm$ 2.52 ^b	-1.03 $\pm$ 0.78 ^d	13.31 $\pm$ 2.16 ^c

*: Mean $\pm$ S.D.

^{a,b,c,d}: Values with different superscripts differ significantly at $P < 0.01$.

Table 5. Photometric values of egg shell color of (RIR-Y 8 $\times$ WL-G) F₁ and (RIR-Y 8 $\times$ WL-r 2) F₁

Mate male $\times$ female	No. of eggs	L-value (brightness)	a-value (red component)	b-value (yellow component)
(RIR-Y 8 $\times$ WL-G) F ₁	244	76.60 $\pm$ 4.00 ^a	1.59 $\pm$ 1.61 ^a	16.10 $\pm$ 2.19
(RIR-Y 8 $\times$ WL-r 2) F ₁	42	69.91 $\pm$ 4.12 ^b	2.59 $\pm$ 1.83 ^b	16.39 $\pm$ 1.95

^{a,b}: Values with different superscripts differ significantly at $P < 0.01$.

significant ($P < 0.01$). Compared with three values of egg shell color between (RIR-Y 8 $\times$ WL-G) F_1 and (RIR-Y 8 $\times$ WL-r 2) F_1 the differences of L and a value were significant ($P < 0.01$) (Table 5). Discriminate functions were calculated after Z-transformation using the three values of egg shell color in the successive four generations. The Z value of one WL-r 4 female exceeded the mean Z value of F_1 (WL-G $\times$ RIR-Y 8).

Discussion

Results of our experiments have clearly shown the success of genetic transformation in the chicken based on the technique described by PANDEY and PATCHELL (1982). The frequency of genetic transformation in this study was 3.8% in WL-r 1. This frequency was almost the same as that of PANDEY's (3.5%). BAUMGARTNER *et al.* (1986) reported a much lower frequency in his study in which 3,646 potential transformation events were screened, and only one apparent transformation has occurred (0.027%). The different frequencies of genetic transformation might be associated with the difference of the chicken strain combinations used. BUMSTEAD *et al.* (1987 a, b) reported that genetic material from irradiated semen was incorporated into the embryo and expressed, but transferred rate was low and cellular mechanism of expression was not clear. SHOFFNER *et al.* (1990) reported that chromatin fragments derived from irradiated spermatozoa was not viable vectors for gene transfer.

KOSIN (1944), SARVELLA (1971) and PANDEY (1980) found that the motility of sperm was significantly decreased as irradiation doses increased from 85 or 100 to 250 Gy. In this paper, we examined the influence of irradiation on chicken semen. The results showed that 1,160 Gy of radiation caused complete destruction of sperm motility. It was possible to recover motility of sperm by 50% by 770 Gy irradiation. Sperm survival rate was decreased greatly after irradiation, and DNA strands in the sperm might be fragmented by these high irradiation doses.

It was observed that semen pH was increased by all doses of irradiation. Generally, motility of sperm was not affected until the pH value was close to 8.5. The pH increase observed in this study should have no effect on sperm motility.

No embryos developed in eggs from females inseminated with only irradiated sperm which had received 370, 770 and 1160 Gy. These results agreed with the previous result of KOSIN (1944).

It was suggested that ionising radiation effectively destroyed sperm fertile ability. On the basis of the results, we decided the irradiation dose of 770 Gy for attaining successful genetic transformation in chicken while avoiding possible fertilization by irradiated sperm.

Usually, sperms penetrate the egg through the vitelline membrane. Then fertilization occurs, and embryos develop normally. On the other hand, irradiated sperm may penetrate the egg, but fertilization does not take place. There are also ample evidence from observations on a variety of animals that sperm treated with relatively high doses of irradiation sufficient for DNA pulverization may maintain their motility and

may be capable of pseudofertilization (HERTWIG 1911). Therefore, if pseudofertilized egg are capable of undergoing a second insemination, it might be possible to use the egg transformation technique in animals including avian species. Production of transgenic chickens seems to require the second insemination with normal unirradiated semen after the first insemination with irradiated semen. It is suggested that the unirradiated sperm penetrated and fertilized the egg.

In this technique integrations of the host DNA should be random. Therefore it is possible that the hatched chicks could have received many genes from RIR-Y 8. However, no chicks showed transmission of other genes from donor sperm than egg shell color in our experiments. We can rule out the possibility of getting a F₁ hybrid in the WL-r 1 generation because we could not observe any change within the limits of plumage color and other outside form in WL-r 1.

In the WL-r 1 generation, 12 of 312 females (3.8%) laid brownish colored eggs. There was no evidence of any color other than white for egg or feather colors in the WL-G. Therefore it is suggested that the brownish color of the egg shell in WL-r 1 could have derived only from the γ -ray irradiated sperm of RIR-Y 8.

Production of transgenic chicken using γ -ray irradiated sperm has some disadvantages; incorporation of the host DNA is random, and irradiation of semen requires expensive facilities. But more than 200 progeny can be obtained per year and hen in layer chickens. When transgene related to egg shell color of WL-r 1 is transmitted from generation to generation, it is possible to establish a transgenic chicken strain. In our experiment, from WL-r 1 to WL-r 4, the transgene related to egg shell color of RIR-Y 8 has been transmitted steadily and even increasingly.

The egg shell color is determined by a number of genes. It is known that brown color is dominant over white (McDONALD 1959, HUNTON 1962). Not all egg shell color genes were transmitted to the individual transgenic chick. But we can integrate these genes by selective matings in the following generations. Actually, WL-r 4 laid the most brownish colored shell eggs among the all successive generations.

In this studies, only egg shell color genes were available to prove gene transformation of RIR-Y 8 genomes to WL-G. It is possible that some genes other than egg shell color genes were integrated simultaneously.

The present results showed that genetic transformation using γ -ray irradiated sperm is possible in chicken. Using this technique new chicken strains can be established by introducing useful genes. Disease resistance genes can also be introduced without changing genetic peculiarities of a strain. Moreover, new information may be obtainable concerning the mechanisms of fertilization, and integration and expression of foreign genes in the host genetic background.

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ガンマー線照射精子による遺伝子導入ニワトリの卵殻色

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本研究は、ニワトリにおいてガンマー線を照射したドナー系統の精子を用いて、レシピエント系統ヘドナー系統がもつ卵殻色遺伝子の導入を試みた。まず、ドナー系統であるロードアイランドレッド種 Y 8 系 (RIR-Y 8) の精子にコバルト 60 を線源とするガンマー線 770 Gy を照射し、レシピエント系統のホワイトレグホーン種 G 系 (WL-G) の雌に人工授精した。次いで 24 時間後、WL-G の非照射正常精子を人工授精した。

その結果、77 羽の WL-G 雌より合計 956 個の卵が得られ、その内の 714 個が孵化 (孵化率 75%, 雄 378 羽, 雌 336 羽) した。孵化した雛を遺伝子導入系第 1 世代 (WL-r1) とした。産卵を開始した WL-r1 雌 312 羽の中で 12 羽 (3.8%) が着色卵を産卵した。WL-r1 の産卵性能は WL-G との比較においても差はなかった。この第 1 世代以降は選択交配を行い次世代を作出した結果、着色卵を産卵した個体は WL-r2 で 8/297 (2.7%), WL-r3 で 8/74 (10.8%), WL-r4 で 19/62 (30.6%) であった。卵殻色の測定には測色色差計を用い、L 値 (明るさ), a 値 (赤色), b 値 (黄色) の 3 成分を測定

した。WL-r4 における卵殻色の平均値は他の世代に比較して、より着色程度が高いものであった。WL-G 系統においてはこれまで着色卵の産卵は確認されていなく、また、WL-r1 ではホワイトレグホーン種とロードアイランドレッド種との F₁ で見られるような羽毛形質は確認されなかった。これらのことから、WL-r 系での卵殻色形質は RIR-Y 8 の照射精子由来である可能性を示唆するものであると考えられる。

本研究で用いた遺伝導入技術は、目的とする遺伝子へのみの導入が困難であるために、形質の発現をもとに選択交配をおこなっていく必要がある。しかしながら、ニワトリの高い繁殖性を利用することで、比較的短期間で特定の質的遺伝子の導入を目的とした育種改良が行える可能性を持つものである。今後、ドナー照射精子由来 DNA の確認などが望まれる。

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