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Research Paper

Development and evaluation of rice giant embryo mutants for high oil content originated from a high-yielding cultivar 'Mizuhochikara'

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Rice bran oil is a byproduct of the milling of rice (*Oryza sativa* L.). It offers various health benefits and has a beneficial fatty acid composition. To increase the amount of rice bran as a sink for triacylglycerol (TAG), we developed and characterized new breeding materials with giant embryos. To induce mutants, we treated fertilized egg cells of the high-yielding cultivar 'Mizuhochikara' with *N*-methyl-*N*-nitrosourea (MNU). By screening M_2 seeds, we isolated four giant embryo mutant lines. Genetic analysis revealed that the causative loci in lines MGE12 and MGE13 were allelic to *giant embryo* (*ge*) on chromosome 7, and had base changes in the causal gene *Os07g0603700*. On the other hand, the causative loci in lines MGE8 and MGE14 were not allelic to *ge*, and both were newly mapped on chromosome 3. The TAG contents of all four mutant lines increased relative to their wild type, 'Mizuhochikara'. MGE13 was agronomically similar to 'Mizuhochikara' and would be useful for breeding for improved oil content.

Key Words: rice bran oil, giant embryo, triacylglycerol (TAG), *N*-methyl-*N*-nitrosourea (MNU), mutant.

Introduction

Rice bran is a byproduct of the rice milling process and consists mainly of the aleurone layer and the embryo. It is rich in protein, oil, and carbohydrates (Das *et al.* 2012, Orthoefer 2005). It is used in the manufacture of animal feed, biofuel, and edible oil. Rice bran oil offers various health benefits (Cicero and Gaddi 2001, Lerma-Garcia *et al.* 2009) and has a beneficial fatty acid composition (Choudhury and Juliano 1980). Crude rice bran oil before being refined includes the following components: triacylglycerol (TAG) (66–77%), diacylglycerol (2.4–3.6%), monoacylglycerol (4.7–6.2%), free fatty acid (1.6–3.6%), waxes (2.4–3.6%), glycolipids (5.4–6.7%), phospholipids (3.6–4.8%), and unsaponifiable lipids (4.2%) (De Deckere and Korver 1996, Sayre and Saunders 1990). Despite a high demand for rice bran oil in Japan, the supply of rice bran has fallen owing to a fall in the consumption of milling rice (MAFF 2009).

To increase the amount of rice bran as a sink for oils, Matsuo *et al.* (1987) proposed breeding for giant embryos, thickening of the aleurone layer, and enlargement of the surface area per volume of brown rice by increasing the number of short seeds. We have focused on the use of giant embryos. Satoh and Omura (1981) doubled to tripled embryo size by treatment of fertilized egg cells with *N*-methyl-*N*-nitrosourea (MNU). Satoh and Iwata (1990) mapped the gene responsible, called *giant embryo* (*ge*) on chromosome 7 by trisomic analysis. Kim *et al.* (1991) also induced giant embryo mutants by MNU treatment of a Korean *japonica* cultivar, 'Hwacheongbyeo'. Molecular mapping located the gene responsible also on chromosome 7 (Koh *et al.* 1996). Molecular cloning studies have shown that this gene encodes a cytochrome P450 and is essential for controlling the embryo/endosperm size balance and for maintenance of the shoot apical meristem (Nagasawa *et al.* 2013, Yang *et al.* 2013). Breeders in Japan produced the first giant embryo cultivar, 'Haiminori' (Maeda *et al.* 2001), followed by 'Mebaemochi', 'Koiazusa', and 'Haiibuki', which are grown as functional foods (Endo *et al.* 2006, Ishii *et al.* 2013, Matsushita *et al.* 2008, Uehara *et al.* 2003).

The purpose of this study was to develop lines with the giant embryo phenotype for use in breeding for high oil content. The high-yielding cultivar 'Mizuhochikara' was used as the genetic background with the expectation that the high-yielding trait would contribute to increased production of

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bran. To induce mutants with giant embryos, we treated fertilized egg cells of 'Mizuhochikara' with MNU, and performed genetic analysis to reveal the underlying genetic mechanisms. We also compared the oil content and agronomic traits of the mutant lines with those of 'Mizuhochikara'.

Materials and Methods

Plant materials, induction of giant embryo mutants, and breeding procedures

'Mizuhochikara', a lodging-tolerant, high-yielding cultivar developed at the National Agricultural Research Center for Kyushu Okinawa Region, Japan, is grown for rice bread and animal feed in southwestern Japan (Sakai *et al.* 2010). To induce giant embryo mutants, we treated fertilized egg cells of 'Mizuhochikara' with MNU (Satoh and Omura 1979, Satoh *et al.* 2010). About 7,000 M_1 seeds were sown, and M_2 seeds from 5,645 M_1 plants were harvested individually. We dehulled these seeds, screened them by eye for giant embryo mutants. M_2 or M_3 plants were backcrossed to 'Mizuhochikara' to remove undesirable traits (differences in traits such as plant type, fertility, and heading date), and we used promising plants of their F_1 to F_4 progeny for phenotypic selection, evaluation of agronomic traits, and measurement of oil content (**Fig. 1**). In addition, we used M_3 plants for allelic testing and mapping and M_4 seeds for the measurement of oil content (**Fig. 1**).

For allelic testing and sequence analysis, the giant embryo mutant EM40, which carries *ge* induced in a *japonica* cultivar, 'Kinmaze', was used as a tester alongside the M_3 plants (**Fig. 1**). For bulked segregant analysis and individual linkage mapping, we used F_2 populations derived from crosses of M_3 plants with a *japonica* cultivar, 'Taichung 65' (**Fig. 1**).

Allelic testing

To test for allelism between the newly induced giant embryo mutants and *ge*, we performed reciprocal crosses among EM40 and four M_3 lines (**Fig. 1**). The tops of husks were not cut in crossing so as to obtain whole hybrid seeds for evaluation of embryo size by eye.

Sequence analysis

PCR primers (forward, 5′-TGGAGGTGTTCGTCGGAAAG-3′; reverse, 5′-CTTTCCGACGAACACCTCCA-3′) were designed to amplify the whole of the *Os07g0603700* gene and were used for sequence determination. *Os07g0603700* lies on chromosome 7, is allelic to the *ge* mutant, and encodes a sequence with homology to a cytochrome P450 (Nagasawa *et al.* 2013). The DNA of

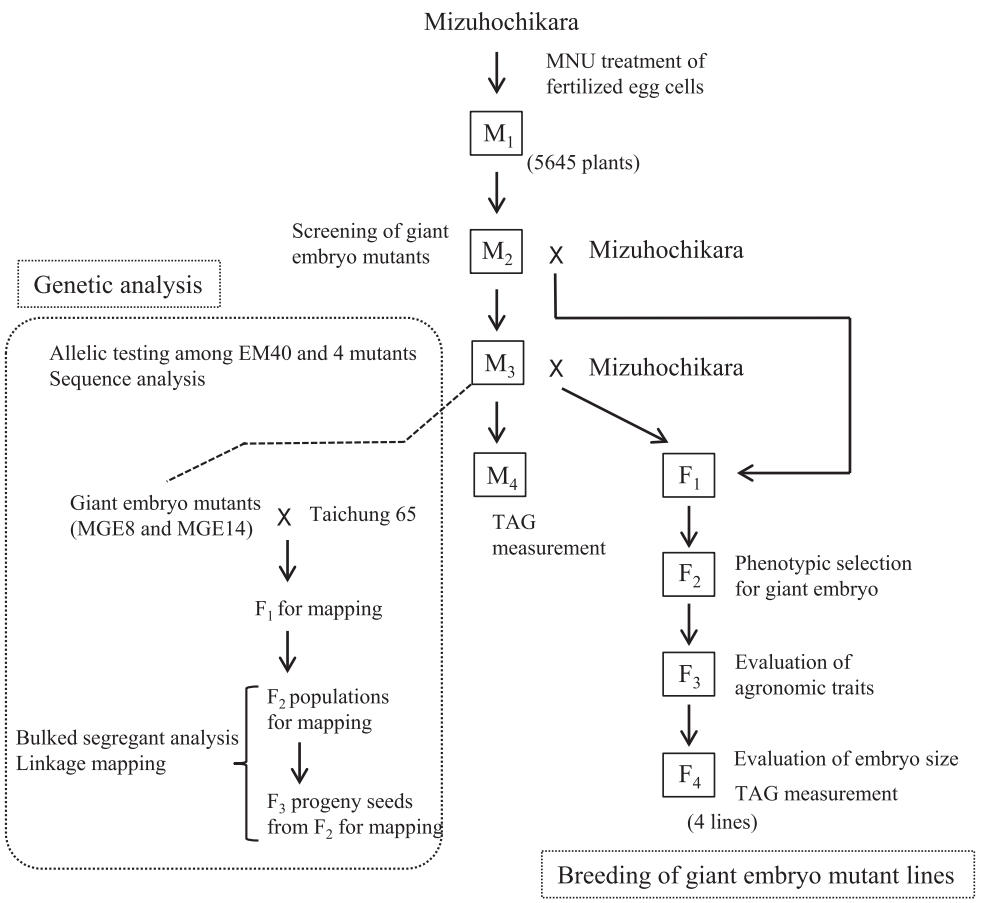

Fig. 1. Breeding scheme for the development of giant embryo mutant lines and materials used for genetic analysis.

Os07g0603700 from giant embryo mutants was sequenced with a BigDye Terminator v. 3.1 cycle sequencing kit and analyzed with a 3130x1 Genetics Analyzer (Applied Biosystems, Foster City, CA, USA). The sequencing data were aligned to the genomic sequences of *Oryza sativa* ssp. *japonica* 'Nipponbare' in Sequencher software (Gene Codes, Ann Arbor, MI, USA).

Genotyping

For genotyping with simple sequence repeat (SSR) markers, we extracted total crude genomic DNA from freeze-dried samples ground in a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) using the method of Dellaporta *et al.* (1983) with minor modifications. We used publicly reported rice SSR markers (IRGSP 2005, McCouch *et al.* 2002, Temnykh *et al.* 2000). PCR reactions were performed in 15-μL mixtures containing 50 mM KCl, 10 mM Tris·HCl (pH 9.0), 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.2 μM each primer, 0.75 units of Taq polymerase (Takara, Otsu, Japan), and approximately 25 ng of template DNA in a GeneAmp PCR system 9700 (Applied Biosystems). PCR conditions were 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. PCR products were run in 4% agarose gels (Agarose HT, Amresco Inc., Solon, OH, USA) in 0.5× TBE buffer.

Linkage analysis

We used bulked segregant analysis to search for markers linked to the target gene (Michelmore *et al.* 1991). Using F_3 progeny seeds from the F_2 populations derived from 'Taichung 65' and the mutant lines, we divided each F_2 population into three genotype groups by eye: giant embryo, segregating, and normal. We prepared DNA pools from five F_2 plants per group and, using 'Taichung 65' and 'Mizuhochikara' as controls, surveyed 71 polymorphic SSR markers screened from 1,207 SSR markers. For individual linkage mapping, we used 64 F_2 individuals to map the giant embryo gene originating from mutant line MGE8 and 86 F_2 individuals to map that from MGE14, using SSR markers *RM14506* and *RM14587* on chromosome 3. Recombination values were calculated by the maximum-likelihood method (Allard 1956) and converted to map distance by the Kosambi map function (Kosambi 1943).

Measurement of triacylglycerol (TAG)

TAG which is main component of rice bran oil was analyzed in this study. For measurement of TAG, we used the four giant embryo mutants in the M_4 seeds, F_4 seeds, and 'Mizuhochikara' as control (**Fig. 1**). After harvesting and drying the seeds of each line, these seeds were kept at constant temperature and humidity. Then, we dehulled the seeds before analysis. In the M_4 seeds, we analyzed the embryo, endosperm, and aleurone layer (including pericarp and seed coat) separately. First, we removed the embryos of 50 brown rice grains per plant with forceps, and then polished the rest of the grain to a 90% yield in a small rice mill (Kett, Tokyo, Japan). The polished grain (endosperm) was then milled finely. In the F_4 seeds, we analyzed the embryo and aleurone together as bran, which we obtained by milling 50 whole brown rice grains.

The lipids were extracted from each tissue by the method of Folch *et al.* (1957). Each tissue was homogenized in 50 mL of chloroform–methanol (2:1, v/v). The mixture was filtered through filter paper (Toyo No. 2) into a graduated cylinder and the filtrate volume was recorded. We then added 10 mL of deionized water to the cylinder, mixed the contents lightly, and allowed the mixture to separate into two layers. The lipid–chloroform layer was collected in a round-bottom flask, and the solvent was removed under reduced pressure and a nitrogen stream in a rotary evaporator at 40°C. The lipid samples were transferred into 10-mL volumetric flasks containing hexane and stored at –20°C until analysis. The TAG was measured with enzyme assay kits (Triglyceride E test; Wako Pure Chemicals, Osaka, Japan): three times in the M_4 generation and five times in the F_4 generation of each line.

Measurement of embryos

We measured embryos of F_4 seeds of the four mutant lines and 'Mizuhochikara'. Dehulled grains were soaked more than 24 hours in 70% ethanol at room temperature to soften. The grains were then cut longitudinally with a razor blade and the cross-sections were photographed. We measured embryo length, width, and area and seed area (endosperm area plus embryo area) in ImageJ v. 1.4.3 software. The embryo ratio was calculated as the embryo area divided by the seed area. Means of embryo traits were calculated from 5 plants of each line and each measured value was from the average of 10 seeds per plant.

Evaluation of agronomic traits

To evaluate whether the mutants retained the superior agronomic traits of 'Mizuhochikara', we evaluated 11 traits (plant height, culm length, panicle length, number of panicles per plant, number of grains per panicle, fertility, panicle weight per plant, yield per plant, seed length, seed width, and seed thickness) of F_3 plants (**Figs. 1**, **2A–2E**). Single plants were transplanted at a spacing of 30 cm × 15 cm in a paddy field at Kyushu University in 2014. The slow release fertilizer with N-P-K at 80-48-40 kg/ha was applied as basal application 10 days before transplanting. Plants in the middle of each row were measured. Means of the first 7 traits were calculated from 10 plants of each mutant and 30 of 'Mizuhochikara'. Means of yield per plant were calculated from 5 plants of each line. Means of seed traits were calculated from 5 plants of each line.

We measured plant height, culm length, panicle length, number of grains per panicle, and fertility as the average values of the three tallest tillers below the tallest one. Plant height was measured from the soil surface to the panicle tip. Culm length was measured from the soil surface to the panicle neck. Panicle length was calculated as the difference.

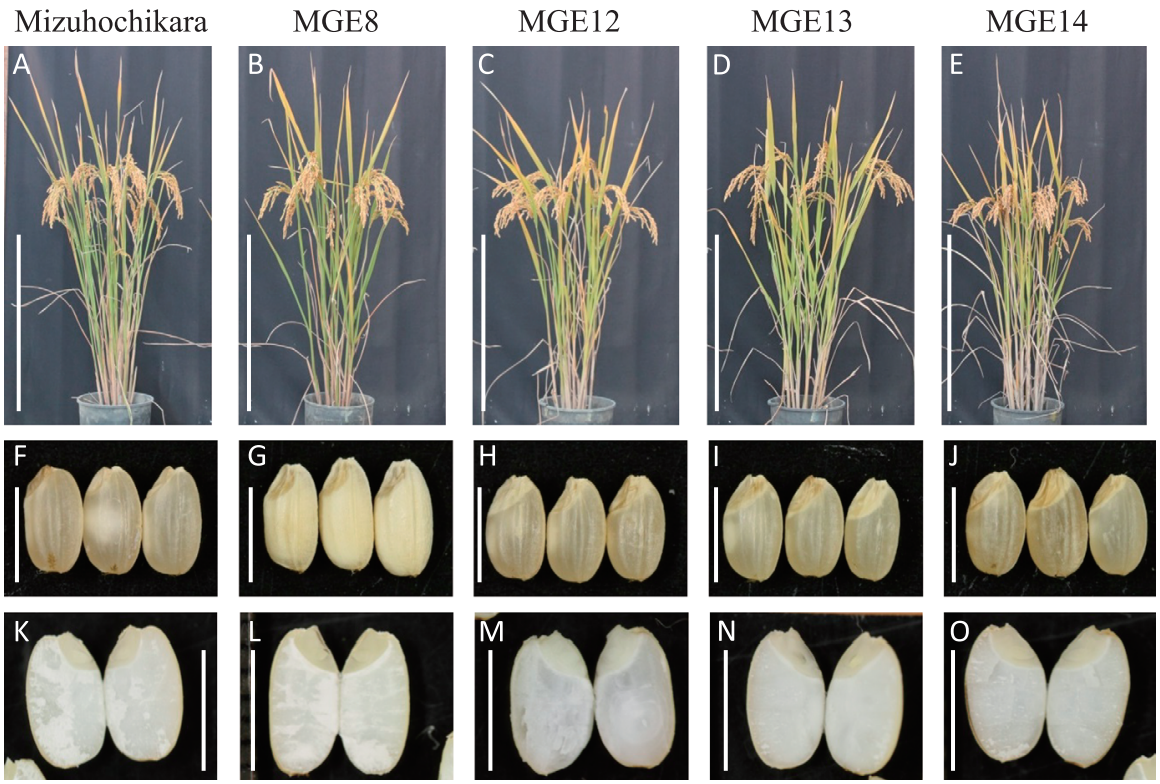

Fig. 2. Phenotypes of giant embryo mutant lines. (A–E) Plant types of 'Mizuhochikara' and four giant embryo mutant lines in the F_3 generation. *Scale bars* = 50 cm. (F–J) Brown rice of 'Mizuhochikara' and mutant lines in the F_4 generation. *Scale bars* = 5 mm. (K–O) Cross-sections of 'Mizuhochikara' and mutant seeds in the F_4 generation. *Scale bars* = 5 mm.

The number of effective panicles per plant was counted and used as the number of panicles. The number of grains per panicle was the total of filled and unfilled grains. Fertility was calculated as the number of filled grains divided by the total number of grains per panicle. Panicle weight was the weight of all panicles per plant. Yield per plant was calculated as (No. of panicles/plant) × (No. of grains/panicle) × (Fertility) × (Weight of brown rice in 50 seeds)/50. To measure seed length and width, 10 seeds per plant were photographed and analyzed in ImageJ software. The thickness of 10 seeds per plant was measured with a dial caliper (Kori Seiki, Tokyo, Japan).

Results

Isolation of giant embryo mutant lines

M_2 seeds obtained from 5,465 M_1 plants showed embryo size variation. From the resultant lines, we phenotypically and genetically confirmed and isolated 4 mutant lines with large embryos in the M_3 generation: MGE8, MGE12, MGE13, and MGE14. The giant embryo phenotype in each line segregated as a single recessive gene in the F_2 populations derived from the crosses between mutant lines and 'Mizuhochikara' (**Supplemental Table 1**).

The embryo length, width, area, and ratio in the four mutant lines were significantly greater than those of 'Mizuhochikara'. The seed area in MGE12, MGE13, and MGE14

Table 1. Embryo size in F_4 seeds of giant embryo mutant lines

Line	Embryo length (mm)	Embryo width (mm)	Embryo area (mm^2)	Seed area (mm^2)	Embryo ratio[a] (%)
MGE8	2.36 ± 0.04**	1.16 ± 0.03**	2.07 ± 0.08**	14.63 ± 0.26^{ns}	14.2 ± 0.4**
MGE12	2.57 ± 0.05**	1.14 ± 0.03**	2.11 ± 0.09**	14.55 ± 0.25^{ns}	14.5 ± 0.5**
MGE13	2.61 ± 0.08**	1.15 ± 0.03**	2.13 ± 0.12**	14.64 ± 0.44^{ns}	14.5 ± 0.6**
MGE14	2.54 ± 0.08**	1.15 ± 0.02**	2.15 ± 0.10**	14.75 ± 0.27^{ns}	14.5 ± 0.5**
Mizuhochikara	2.10 ± 0.06	0.90 ± 0.04	1.41 ± 0.08	14.92 ± 0.31	9.4 ± 0.4

Values are means ± SD of 5 replicates of about 10 seeds each. Dunnett's test was performed for each trait using 'Mizuhochikara' as the reference.
** $P < 0.01$; 'ns', not significant at $P = 0.05\%$.
[a] Embryo ratio = embryo area/seed area.

Table 2. Allelic testing of giant embryo mutant lines

Cross combination		Embryo phenotype of F_1 seeds
Female	Male	
EM40	MGE8	Normal
EM40	MGE12	Giant embryo
EM40	MGE13	Giant embryo
EM40	MGE14	Normal
MGE8	EM40	–
MGE12	EM40	Giant embryo
MGE13	EM40	Giant embryo
MGE14	EM40	Intermediate size
MGE12	MGE13	Giant embryo
MGE13	MGE12	Giant embryo
MGE8	MGE14	Intermediate size
MGE14	MGE8	Intermediate size

was not significantly different (**Table 1**, **Fig. 2F–2O**). MGE8 showed a different shape (**Fig. 2G**, **2L**), but the seed area was not significantly different (**Table 1**). The brown rice of MGE8 had floury endosperm (**Fig. 2G**), but whether this is related to the giant embryo phenotype is unclear.

Allelic testing of giant embryo mutants

For allelic testing, the giant embryo mutant EM40, which carries *ge* induced in a *japonica* cultivar, 'Kinmaze', was used as a tester line. The F_1 seeds from reciprocal crosses among EM40, MGE12, and MGE13 showed the giant embryo phenotype, indicating that the loci in MGE12 and MGE13 were identical to *ge* (**Table 2**). On the other hand, the F_1 seeds of EM40 × MGE8 and EM40 × MGE14 showed normal embryos and those of MGE14 × EM40 did not show a typical giant embryo phenotype, suggesting that neither locus was allelic to *ge*. As the F_1 seeds of MGE14 × EM40 did not show a typical giant embryo or normal embryo phenotype, we described the phenotype as intermediate size. The difference of reciprocal crosses between EM40 and MGE14 is unclear. As the embryo size of MGE8 × MGE14 was intermediate, their allelic relationship was not clear.

Sequencing analysis

We found sequence differences within *Os07g0603700* in EM40, MGE12, and MGE13 relative to the 'Nipponbare' reference sequence (**Fig. 3**). Base changes between 'Nipponbare' and 'Kinmaze' in the *Os07g0603700* ORF sequence were detected at nucleotide positions 1105 and 1317. There was a nonsense mutation at nucleotide position 1482 in EM40. The mutation found in EM40 is consistent with that of a previous report (Nagasawa *et al.* 2013). There were missense mutations at nucleotide positions 994 in MGE12 and 1180 in MGE13. No base changes in *Os07g0603700* were detected in MGE8 and MGE14. Thus, the causal gene of the two mutants MGE12 and MGE13 might be *Os07g0603700*, and those of MGE8 and MGE14

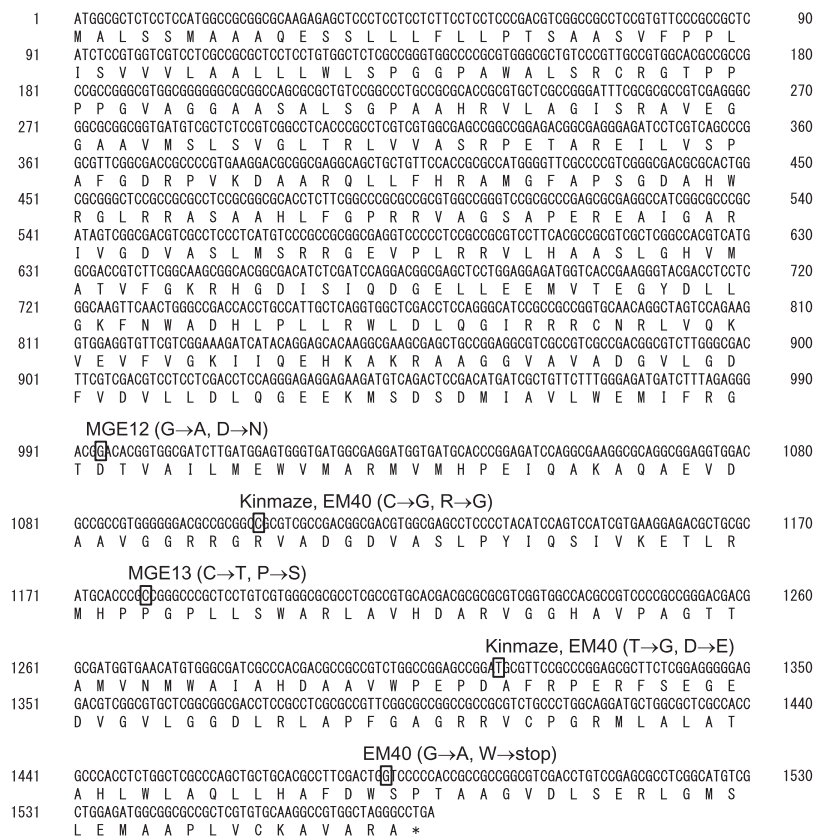

Fig. 3. Mutations in *Os07g0603700* of EM40, MGE12, and MGE13. Boxes show base changes relative to 'Nipponbare' reference sequence.

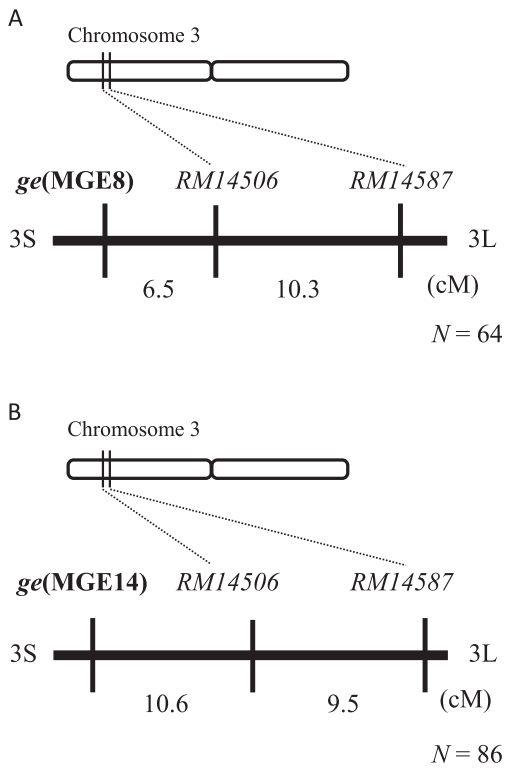

Fig. 4. Linkage maps of (A) *ge*(MGE8) and (B) *ge*(MGE14) on chromosome 3.

were different. This result accords with the result of the allelic testing.

Bulked segregant analysis and mapping of new genes in MGE8 and MGE14

Out of the 71 SSR markers that were polymorphic between 'Taichung 65' and 'Mizuhochikara', *RM14584*, on chromosome 3, was apparently linked with the mutant loci in MGE8 and MGE14. Both loci were mapped on the short arm of chromosome 3 (**Fig. 4**). Since genes for giant embryo have not previously been identified in that region, the giant embryo genes in MGE8 and MGE14 are new, but it is not clear whether loci in MGE8 and MGE14 are allelic.

TAG measurement of four mutants

The TAG contents of MGE12, MGE13, and MGE14 in embryos were more than double compared to 'Mizuhochikara', but that in MGE8 was not significantly different (**Table 3**). The TAG contents in aleurone of MGE12 and MGE13 were significantly higher than that of 'Mizuhochikara'. The TAG contents in endosperm of four mutants were not significantly different from that of 'Mizuhochikara'

The quantity of TAG in embryos showed no significant difference among MGE12, MGE13, MGE14, and 'Mizuhochikara', but was significantly lower in MGE8 (**Table 4**). The quantity of TAG in aleurone of MGE12, MGE13, and MGE14 was significantly higher than that of 'Mizuhochikara', but

Table 3. TAG contents of each tissue in M_4 seeds of giant embryo mutant lines

Line	TAG contents of each tissue (mg/50 seeds)				
	Embryo	Aleurone[a]	Endosperm	Rice bran[b]	Brown rice[c]
MGE8	5.3 ± 0.7^{ns}	9.6 ± 0.6^{ns}	3.2 ± 0.2^{ns}	14.9 ± 0.3^{ns}	18.1 ± 0.1*
MGE12	10.9 ± 0.2**	11.7 ± 1.0**	3.0 ± 0.1^{ns}	22.5 ± 1.2**	25.6 ± 1.2**
MGE13	8.8 ± 1.0**	11.3 ± 0.9*	3.3 ± 0.4^{ns}	20.1 ± 1.9**	23.4 ± 2.2**
MGE14	11.4 ± 0.5**	9.8 ± 1.2^{ns}	3.2 ± 1.5^{ns}	21.1 ± 0.7**	24.4 ± 0.8**
Mizuhochikara	4.1 ± 0.3	8.5 ± 0.5	2.2 ± 0.1	12.6 ± 0.8	14.9 ± 0.8

Values are means ± SD of 3 replicates. Dunnett's test was performed for TAG contents using 'Mizuhochikara' as the reference. * $P < 0.05$, ** $P < 0.01$; 'ns', not significant at $P = 0.05$%.

[a] Aleurone includes pericarp and seed coat.

[b] Rice bran consists of embryo and aleurone.

[c] Brown rice consists of embryo, aleurone, and endosperm.

Table 4. Quantity of TAG per gram of tissue in M_4 seeds of giant embryo mutant lines

Line	Quantity of TAG per 1 g of tissue (mg/g)				
	Embryo	Aleurone[a]	Endosperm	Rice bran[b]	Brown rice[c]
MGE8	150.2 ± 7.3**	89.6 ± 19.0^{ns}	6.4 ± 0.6**	103.7 ± 16.3^{ns}	28.0 ± 1.4**
MGE12	201.9 ± 17.0^{ns}	99.5 ± 11.6*	3.3 ± 0.2^{ns}	131.7 ± 13.0**	23.3 ± 1.1**
MGE13	189.8 ± 11.0^{ns}	113.1 ± 2.4**	3.9 ± 0.3^{ns}	137.3 ± 2.0**	23.4 ± 1.4**
MGE14	221.2 ± 17.0^{ns}	102.7 ± 9.5*	4.0 ± 2.0^{ns}	144.0 ± 8.8**	25.4 ± 1.4**
Mizuhochikara	194.9 ± 3.6	72.8 ± 0.5	2.2 ± 0.1	91.4 ± 1.5	12.9 ± 0.4

Values are means ± SD of 3 replicates. Dunnett's test was performed for TAG contents using 'Mizuhochikara' as the reference. * $P < 0.05$, ** $P < 0.01$; 'ns', not significant at $P = 0.05$%.

[a] Aleurone includes pericarp and seed coat.

[b] Rice bran consists of embryo and aleurone.

[c] Brown rice consists of embryo, aleurone, and endosperm.

Table 5. TAG contents and weight of rice bran in F_4 seeds of giant embryo mutant lines

Line	TAG contents of rice bran in 50 seeds (mg)	Quantity of TAG per g of rice bran[a] (mg)	Quantity of TAG per g of brown rice[b] (mg)	Weight of rice bran in 50 seeds (g)	Weight of brown rice in 50 seeds (g)	Rice bran/brown rice ratio (%)
MGE8	15.6 ± 1.0**	73.5 ± 4.3**	22.0 ± 1.2**	0.213 ± 0.003**	0.709 ± 0.018**	30.0
MGE12	18.8 ± 0.4**	141.1 ± 8.2^{ns}	19.2 ± 0.4**	0.133 ± 0.006**	0.978 ± 0.010**	13.6
MGE13	19.7 ± 0.7**	160.4 ± 3.2**	19.7 ± 1.2**	0.123 ± 0.005**	1.001 ± 0.031**	12.3
MGE14	21.2 ± 0.8**	166.5 ± 9.6**	22.2 ± 0.8**	0.128 ± 0.008**	0.956 ± 0.029**	13.4
Mizuhochikara	13.9 ± 0.7	132.7 ± 3.6	13.0 ± 0.3	0.105 ± 0.005	1.070 ± 0.035	9.8

Values are means ± SD of 5 replicates (4 replicates of MGE8). Dunnett's test was performed using 'Mizuhochikara' as the reference.
** $P < 0.01$; 'ns', not significant at $P = 0.05$%.
[a] Calculated as (TAG content of rice bran in 50 seeds)/(weight of rice bran in 50 seeds).
[b] Calculated as (TAG content of rice bran in 50 seeds)/(weight of brown rice in 50 seeds).

Table 6. Agronomic traits of giant embryo mutant lines in F_3 plants

Line	Plant height (cm)	Culm length (cm)	Panicle length (cm)	No. of panicles/ plant	No. of grains/ panicle	Fertility (%)	Panicle weight/plant (g)	Yield/plant[a] (g)	Seed length (mm)	Seed width (mm)	Seed thickness (mm)
MGE8	78.9 ± 1.9^{ns}	56.1 ± 1.4**	22.7 ± 0.8**	10.9 ± 2.5^{ns}	241.3 ± 31.4**	85.6 ± 4.3**	30.7 ± 6.6**	37.7 ± 4.7^{ns}	8.13 ± 0.13**	3.37 ± 0.02^{ns}	1.95 ± 0.04**
MGE12	80.3 ± 3.5^{ns}	58.2 ± 3.1^{ns}	22.1 ± 1.3**	10.8 ± 2.0^{ns}	212.3 ± 34.0^{ns}	88.5 ± 3.0*	37.4 ± 8.4^{ns}	47.4 ± 8.6^{ns}	7.43 ± 0.09^{ns}	3.54 ± 0.04**	2.30 ± 0.03^{ns}
MGE13	82.7 ± 3.5^{ns}	62.6 ± 3.0**	20.1 ± 1.0^{ns}	10.7 ± 2.0^{ns}	191.6 ± 30.1^{ns}	92.8 ± 2.4^{ns}	38.3 ± 8.4^{ns}	48.7 ± 8.7^{ns}	7.42 ± 0.07^{ns}	3.41 ± 0.04^{ns}	2.26 ± 0.02^{ns}
MGE14	74.1 ± 2.8**	55.7 ± 2.3**	18.4 ± 0.9**	9.9 ± 2.2^{ns}	223.7 ± 24.8*	90.6 ± 2.9^{ns}	35.3 ± 9.2^{ns}	46.6 ± 7.2^{ns}	7.51 ± 0.09^{ns}	3.43 ± 0.06^{ns}	2.16 ± 0.03**
Mizuhochikara	80.2 ± 3.7	59.4 ± 3.3	20.8 ± 0.9	10.9 ± 1.7	199.1 ± 16.6	91.2 ± 2.3	41.4 ± 8.1	44.4 ± 4.9	7.50 ± 0.09	3.41 ± 0.03	2.28 ± 0.04

Values are means ± SD. Data in first 7 columns were measured with 10 replicates for giant embryo mutant lines and 30 replicates for 'Mizuhochikara'. Yield data were calculated from 5 replicates. Seed data were measured with 5 replicates. Dunnett's test was performed for each trait using 'Mizuhochikara' as the reference. * $P < 0.05$, ** $P < 0.01$; 'ns', not significant at $P = 0.05$%.
[a] Calculated as (No. of panicles/plant) × (No. of grains/panicle) × (Fertility) × (Weight of brown rice in 50 seeds)/50.

that in MGE8 was not significantly different. The quantity of TAG in endosperm of MGE12, MGE13, and MGE14 was not significantly different from that of 'Mizuhochikara', but MGE8 had significantly more. The quantity of TAG in bran of MGE12, MGE13, and MGE14 was significantly higher than that of 'Mizuhochikara', but that of MGE8 was not significantly different. Overall, the quantity of TAG in brown rice was significantly increased in all mutant lines. Embryo had the highest contents, followed by aleurone, in all lines. The contents (**Table 3**) and the quantities (**Table 4**) of TAG in aleurone of MGE12, MGE13, and MGE14 were significantly higher (with one exception) than those of 'Mizuhochikara'. These results suggest that the giant embryo gene influenced not only embryo size, but also oil content in aleurone cells. This result is consistent with a previous report (Matsuo *et al.* 1987).

The quantity of rice bran was also significantly higher in the four mutants than in 'Mizuhochikara' (**Table 5**). Much of the brown rice of MGE8 cracked during milling, and parts of the endosperm were probably mixed in the bran, increasing the quantity of "bran" and decreasing its TAG content. In the other mutants and 'Mizuhochikara', marked cracks were not observed during milling. The pattern of TAG contents in the F_4 seeds (**Table 5**) approximated that in the M_4 seeds (**Table 3**).

Evaluation of agronomic traits

MGE8 had a significantly shorter culm, longer panicle, more grains per panicle, lower fertility, and lighter panicle than 'Mizuhochikara' (**Table 6**). Its seeds were longer and thinner. MGE12 had a significantly longer panicle, lower fertility, and wider seeds. MGE13 was not significantly different except in having a longer culm. MGE14 had significantly shorter plants, culms, and panicles, more grains per panicle, and thinner seeds. Yields per plant of four mutant lines were not significantly different from that of 'Mizuhochikara'. Thus, MGE13 was most similar to 'Mizuhochikara'.

Discussion

In this study, we developed and characterized new plant materials for breeding for high oil content, isolating four giant embryo mutant lines. Genetic analysis revealed that the responsible loci in lines MGE12 and MGE13 were allelic to *ge* on chromosome 7, and had base changes in the causal gene *Os07g0603700*. The responsible loci in MGE8 and MGE14 were not allelic to *ge* and were mapped on the short arm of chromosome 3 (**Fig. 4**). The TAG contents of the mutant lines were greater than those of 'Mizuhochikara'. MGE13 was agronomically most similar to 'Mizuhochikara' and will be useful for breeding new cultivars with high oil content.

The loci in MGE8 and MGE14 were located on the short arm of chromosome 3 (**Fig. 4**). The rice *goliath* mutant, which has an enlarged embryo phenotype, was mapped on the long arm of chromosome 3 (Taramino *et al.* 2003), and *plastochron3*, which is allelic to *goliath*, was located at the *Os03g0790600* locus (Kawakatsu *et al.* 2009). This position

is different from that of the loci in MGE8 and MGE14, so these loci are new. *ge*(MGE8) and *ge*(MGE14) were mapped to same region (**Fig. 4**). Since allelic testing of MGE8 and MGE14 gave an intermediate embryo size (**Table 2**), it is not clear whether *ge*(MGE8) and *ge*(MGE14) are allelic. High-resolution mapping and molecular cloning will be needed to verify their precise position(s). Nagasawa *et al.* (2013) reported that the *GIANT EMBRYO* gene encodes a cytochrome P450, CYP78A13. The CYP78 family is involved in the control of flower and fruit size and of apical meristem development (Mizutani and Ohta 2010, Nelson and Werck-Reichhart 2011). Therefore, *ge*(MGE8) and *ge*(MGE14) may be related to a cytochrome P450. MGE8 and MGE14 will prove useful for studying embryogenesis and analyzing the molecular mechanisms of interactions between embryo and endosperm.

In general, endosperm contamination of rice bran due to over-milling dilutes the TAG content. When the embryo is detached early in the milling process, the projecting parts of the endosperm increase the content of endosperm in the bran. By contrast, when the embryo is detached late in the milling process, less endosperm contaminates the bran. The TAG contents of bran in F_4 seed (**Table 5**) showed a similar tendency to those in the M_4 seed (**Table 3**), but the quantity of TAG in the bran was higher in the F_4 seed (**Tables 4**, **5**), except in MGE8. We suspect that this difference is due to differences in sample preparation between M_4 and F_4 seeds: we separated the embryo from the M_4 brown rice before milling, but we milled the F_4 brown rice whole. This likely increased the quantity of endosperm in the bran of the M_4 rice.

The TAG contents per plant were estimated by multiplying the quantity of TAG per g of brown rice (**Table 5**) by yield per plant (**Table 6**). The estimated TAG contents per plant were 0.829 g in MGE8, 0.911 g in MGE12, 0.959 g in MGE13, 1.035 g in MGE14, and 0.578 g in 'Mizuhochikara'. The increase of the TAG contents of the mutant lines were 144% in MGE8, 158% in MGE12, 166% in MGE13, and 179% in MGE14, as compared with TAG content of 'Mizuhochikara'. Oil production was remarkably increased in the giant embryo mutant lines.

Giant embryo mutants have shown problems in germination and seeding establishment (Maeda *et al.* 2001). In 2014, a low germination rate (approximately 70–80%) and delayed growth of the seedling were observed in all four mutant lines including MGE13. When we sowed many seeds of MGE13 on seed boxes for a transplanting-machine in 2015, such low germinability and weak growth did not affect the normal practice of transplanting by the transplanting-machine. In addition, we recommend testing fatty acid composition and functional components such as phytosterols, γ-oryzanol, and vitamin E to confirm the quality of rice bran oil in new giant embryo cultivars. In this study, we developed giant embryo mutant lines in the 'Mizuhochikara' genetic background. Line MGE13 offers the best promise for breeding for increased oil production. Further selection of plants, the production of breeding seeds, and yield tests are required for the development of new cultivars.

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