Selection of Novel Non-lethal, Low Phytic Acid Mutants and Evaluation of their Agronomic Traits/Mineral Compositions in Rice (*Oryza sativa*)

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Abstract

In the grains of cereal crops, 65% to 85% of phosphorus is stored as phytic acid, regardless of the crop species. Non-ruminant animals that include humans, cannot digest phytic acid, and they are unable to use most of the phosphorus in grains. And given the chelating ability of phytic acid, some of the nutritionally important minerals that are stored in phytic acid are not assimilated in these animals. Intending to improve the nutritional value of rice grain in three major cultivars in Japan, we obtained six non-lethal low phytic acid mutants in which the phytic acid content ranged from 42.1% to 94.1% relative to that of their original cultivars. Most of the agronomic traits of the six mutants were comparable to those of the original cultivars, except for a slightly weak germination ability in two mutants whose phytic acid contents were the lowest among the six mutants. In the grains of our mutants, the iron content correlated positively and the calcium contents correlated negatively with the phytic acid contents.

Introduction

Phytic acid (*myo*-inositol 1, 2, 3, 4, 5, 6 hexakisphosphate) is a circular carbohydrate molecule with six phosphorus atoms. Cereal crops accumulate it in their grains to store phosphorus and minerals for their germination and early seedling growth. Phytic acid is considered to incorporate several mineral cations in the cereal grains using its chelating ability in order to form stable salts. Regardless of the species, 65% to 85% of phosphorus is stored as phytic acid in cereal grains (Raboy 2002). Non-ruminant animals lack the phytase for phytic acid, and thus are unable to digest phytic acid and use the phosphorus contained in it.

The first low phytic acid (lpa) mutant was obtained in maize by mutant screening (Ertl et al. 1998). Though most of the lpa mutants were lethal, the authors obtained non-lethal lpa mutants and used them for genetic and physiological analysis. After the maize mutants, a number of lpa mutants have been obtained in various crop species. These include barley mutants (Larson et al. 1998), soybean mutants (Wilcox et al. 200), common bean mutants (Panzeri et al. 2011) and rice mutants (Larson et al. 2000).

During the germination process of seeds, phytic acid

is possibly the source of other major minerals. Due to the decreased mineral supply, many lpa mutants reportedly showed weak germination vigor and lethality when their phytic acids contents dropped below a certain critical threshold. Though the lpa mutants offer potential value as fodder feeds or foods, these resultant pleiotropic influences of lpa on common agricultural traits must be evaluated.

We intended to obtain lpa mutants in order to introduce new forage rice cultivars with improved nutritional value for non-ruminant animals, and thus meet the growing demand for domestic forage rice. For this purpose, we applied a mutation induction procedure to our major rice cultivars - *Nipponbare, Koshihikari* and *Dontokoi*. In this report, we analyzed the decrease of phytic acid contents, growth characteristics and the influence of the decrease in phytic acid content on the mineral concentration in novel non-lethal lpa mutants.

Materials and methods

1. Mutagenic treatment

Dried seeds of cultivar *Dontokoi* and *Koshihikari* were treated with 0.1 M ethyl methane sulfonate (EMS) for four

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hours at room temperature. Another dried seeds of Koshi-hikari were irradiated with 200 Sv gamma-rays at 1 Sv/hr in the irradiation facility of Institute of Radiation Breeding, National Institute of Agrobiological Research. These seeds were planted and harvested individually. *Nipponbare* seedlings were regenerated from the calli of an immature embryo culture at the National Institute of Agrobiological Sciences and the seeds of self-pollinated offspring from the regenerant plants were planted and harvested individually.

2. Mutant selection and phenotype confirmation

Phytic acid mutants were screened by identifying any excess amount of free phosphorus in the endosperm using a molybdenum blue method that was slightly modified after Larson et al. (2000), based on the assumption that lpa mutants have an increased amount of free phosphorus that corresponds to the decrease of phytic acid. The brown rice grains were individually crashed and free inorganic phosphorus was extracted with 100 μl 0.4 M HCl in PCR microplates. The extract (20 μl) was mixed with 80 μl distilled water and 100 μl reaction mixture (0.6 M sulfuric acid, 0.05% (w/v) ammonium molybdate, 2% (w/v) ascorbic acid). After 15 to 30 minutes of incubation at room temperature, the panicles whose grain extract showed deep blue coloration were kept for subsequent analyses.

3. Cultivation for yield analysis

After the germination in nursery boxes, the seedlings at the five- to six-leaved stage were transplanted individually in the paddy field. The rice plants were cultivated on basal fertilizer of 2.0 kg nitrogen per 10 a and later supplied 2 kg nitrogen per 10 a as a supplemental fertilizer at the maturity stage. The culm length, panicle length and panicle number of 10 plants in 2007 and 20 plants in three years (2008 to 2010) were measured at maturity stage. The rice grains were harvested from individual plants for genetic analysis. For yield measurement, grains were harvested from more than 150 plants in 2009 and 2010. For yield analysis, air-dried and dehulled grains were sieved through a grain sieve 1.8-mm wide and polished to roughly 90% weight by a small scale-scale polisher. And in 2009, 500 dehulled and sieved brown grains were weighed to measure grain weight.

4. Germination rate and seedling growth estimation

For the observation of germination, 300 seeds for each mutant and cultivar were presoaked in water at room temperature for three days. The seeds were planted on a plastic mesh in plastic Petri dishes and incubated at 25°C with 16 hours of lighting a day. Seeds with germinated seedlings were counted every day for 10 days. For the observation of seedling growth, the seedling height of germinated seeds were measured every day for ten days while the seedlings grew solely on nutrition from their endosperm.

5. Evaluation of chemical components in grains
1) Phytic acid

The phytic acid contents had been analyzed from the 290 to 320 grams of brown grains for four years (2007 to 2010). Phytic acid content was measured at the Japan Inspection Association of Food and Food Industry Environment using the analysis method of the National Food Research Institute (Matsunaga et al. 1990). Five grams of grain sample were homogenized in 30 ml of 4% trichloroacetic acid and 0.1% phosphoric acid. The centrifuged precipitate was homogenized again in the same solution. The supernatant and precipitates were all mixed and made up to 100 ml by the same solution, and then filtered through 0.45 μm ADVANTECH DISMIC-25cs. Phytic acid was separated using HPLC (column: Waters Protein Pak G-QA and TSK-GEK DEAE-5PW, mobile phase: 0.01N nitric acid and 0.25 M sodium nitric acid, reaction solution: 0.5 mM ferric chloride and 0.25 mM sulfosalicylic acid) and detected at 500 nm wavelength.

2) Total phosphorus

To measure the total phosphorus in grains, 1 gram of homogenized grain sample was suspended in 20 ml 1N HCl. After filtration with a filter paper, 0.1 ml solution with 5 ml distilled water was mixed with 1 ml reaction solution ($2.5N$ H_2SO_4, 5 mM ammonium molybdate, 3.5 mM ascorbic acid, 0.2 mM antimonyl potassium tartrate). After one to two hours at room temperature, the blue color of the reacted solution was measured at 710 nm wavelength using a spectrophotometer. The difference between the phosphorus values of phytic acid and total phosphorus was considered as inorganic phosphorus.

3) Mineral components

Mineral components (B, Ca, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, S, Si, Zn) were measured in grains harvested in 2010 by the ICP (inductively coupled plasma) detection system. For the extraction of minerals, 0.25 gram of homogenized grain sample was suspended in 25 ml 1N HCl. The solution was filtered through 0.45 μm ADVANTECH DISMIC-25cs and injected in the Jarrell Ash IRIS Advantage system. The photo spectrum was detected and mineral concentrations were analyzed by ThermoSPEC/CID software. Dr. Mitsuhara at the NARO Agricultural Research Center conducted the statistical analysis of mineral concentrations. After regression analysis of the all the content combinations of minerals, phosphorus and phytic acid, statistically significant regression variances were selected by step-wise multiple regression analysis.

Results

1. Mutant selection

About 3000 panicles of each cultivar were harvested individually. Many segregating panicles showing various

degrees of blue color were found in the initial screening, while the degree of color reflected the amount of free inorganic phosphorus. As we were not interested in mutations exhibiting a slight decrease of phytic acid, we only screened plants showing clear color reactions. Considering all three cultivars, more than fifty mutants showed distinct reactions with deep blue color. However, the seeds of these mutants only showed signs of germination at the germination stage and did not continue their growth anymore. Consequently, non-lethal mutants which showed intermediate blue color reaction were selected.

From this whole series of this mutant screening, six non-lethal mutants were obtained, NC1857 from *Nipponbare*, 176 from *Dontokoi* and 49, 2623, 3847 and 4019 from *Koshihikari*. The plant performance of these mutants was observed over the several generations and undesirable accompanying mutations (e.g. infertility, inferior growth) were screened out by observing individual plants as long as possible. Finally, the mutants with growth phenotypes similar to those of their original cultivars were selected and subjected to further analysis.

Genetic segregation in the mutants was counted to confirm the number of genes involved. The seeds from putative heterozygotes derived from self-pollinated heterozygotes were segregated as shown in Table 1 and the most of the segregation values were fit to a 3:1 single recessive gene segregation. In Dontokoi176 and Koshihikari3847 seeds, slightly more seeds with a mutant phenotype than expected from a single recessive gene had been segregated. However, because deviations of the segregation ratio from the expected single gene segregation ratio in Koshihikari3847 was not large, the segregation ratio was not justified in any two recessive mutant genes model. In Dontokoi176, the deviation from the 3:1 ratio expected from a single recessive gene model was relatively large. Because the color reaction in this mutant varied depending on each grain

due to an unknown reason in this mutant, slightly colored wild type grains might be counted as mutant phenotype grains. However, the segregation ratio was not justified in any two or more recessive mutant genes model. Therefore, we considered that all the six mutants had their own single recessive mutant gene.

2. Growth and yield of the mutants

The culm length, panicle length and panicle number of each plant were measured at the maturity stage for four years (Fig. 1). Relative to their respective original cultivars, Dontokoi176, Koshihikari49 and Koshihikari4019 decreased 85.1% to 89.6%, in terms of culm length, while the other mutants showed only a slight decrease. There was no significant difference in the panicle numbers of the mutants and those of their respective original cultivars.

The grain appearance of brown rice was visually observed (data not shown). Of the original cultivars, *Nipponbare* and *Dontokoi* had clear grains, while *Koshihikari* had clear grains with occasional limited whiteness. All mutants had grains showing an opaque or white appearance, with a different frequency and to a different degree depending on each mutant. NC1857 and Dontokoi176 had relatively clear grains. The grains of Koshihikari2623 and Koshihikari4019 showed a white appearance.

Grain weight was measured for the mature grains of each mutant (Fig. 2). Though the grain weight in NC1857, Koshihikari2623 and Koshihikair3847 was slightly reduced compared to those of their original cultivars, there were no apparent differences in the grain weights between those of the mutants and their respective original cultivars.

The grain yield for hulled grains, gross brown grains and mature brown grains was measured over two years (Fig. 3). The yield per 10 ares of hulled grains was 645.7 kg, 774.7 kg and 713.9 kg for *Nipponbare, Dontokoi* and *Koshihikari*, respectively. The yields of gross brown

Table 1. Mutants and their genetic segregation in 2004 and 2006

Mutants	Mutagen	WT	MT	P (3:1)	Year observed
NC1857	callus culture	138	54	0.317	2004
Dontokoi176	EMS	128	64	0.008 **	2004
Koshihikari49	gamma rays	110	34	0.700	2006
Koshihikari2623	EMS	112	32	0.441	2006
Koshihikari3847	EMS	132	60	0.046 *	2006
Koshihikari4019	EMS	109	35	0.847	2006

WT: number of wild type, MT: number of mutant types
P (3:1) was calculated by chi-square analysis where * or ** indicates values statistically different from the expected value at the 5% or 1% levels respectively.
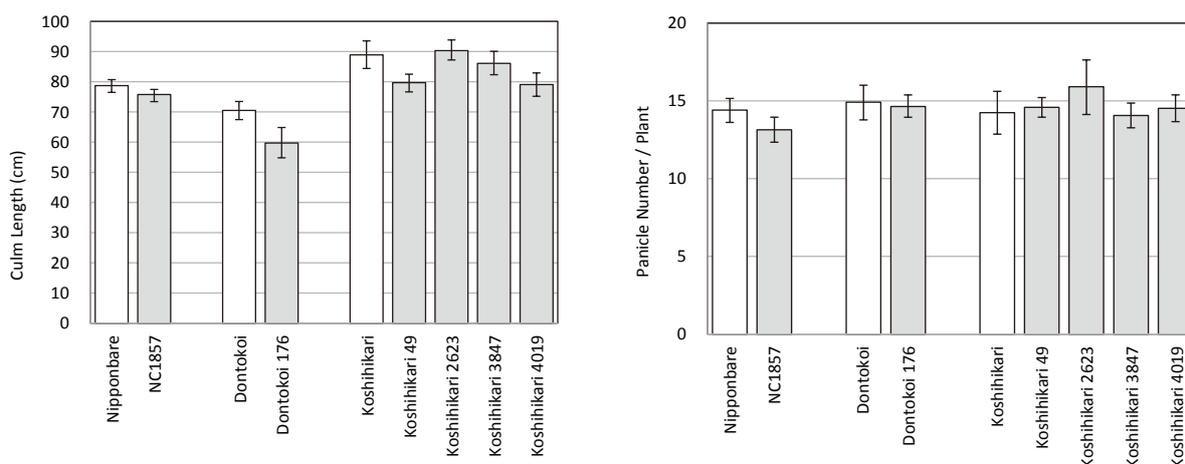
Fig. 1. Culm length (left) and panicle number (right) at the maturity stage
Blank bars indicate the original cultivars; shaded bars indicate the mutants.
The data are the average of observation over four years (2007 to 2010), and the error bars represents the standard deviations.
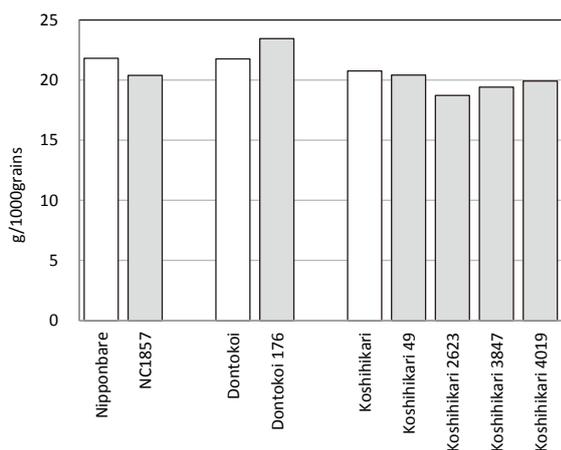

Fig. 2. Grain weight of the mature brown grains in 2009.
Blank bars indicate the original cultivars; shaded bars indicate the mutants.

grains were 81.7% to 82.3% of the yields of hulled grains in the three cultivars, and the yields of mature brown grains were 95.0% to 98.7% of the yields of gross brown grains in the three cultivars. However, in Koshihikari4019, though the yields of hulled grains and gross brown grains were 91.6% and 88.9% those of their original cultivars respectively, the yield of mature brown grains was 57.3% of that of its original cultivar. Koshihikari3847 showed a similar decrease in mature brown grain yield as Koshihikari4019. The specific decrease in the yields of mature brown grains suggests an increase of immature grains. The other four mutants showed 73.0% to 89.0% yield in mature brown grains as compared to those of their original cultivars.

3. Phytic acid and minerals in the endosperm

A molecule of phytic acid contains six phosphorus molecules. Figure 4 shows the phosphorus content in phytic acid, which is proportional to the phytic acid contents, and the inorganic phosphorus content in brown grains of the original cultivars and mutants. The phytic acid content relative to that of their respective original cultivars was 82.9% in NC1857, 85.3% in Dontokoi176, 94.1% in Koshihikari49, 55.3% in Koshihikari2623, 42.1% in Koshihikri3847 and 64.1% in Koshihikari4019.

As for the total phosphorus content, four mutants (NC1857, Koshihikari2623, Koshihikari3847 and Koshi-hikari4019) had almost the same amount of phosphorus as their respective original cultivars. In Dontokoi176, the total phosphorus content increased to 122.0% from that of its original cultivar, whereas in Koshihikari49, total phosphorus content was slightly increased to 109.4% of its original cultivar.

Table 2 lists the mineral concentrations after brown rice grains were subjected to ICP analysis, and indicates increases or decreases in the mineral concentrations in the mutants. In Dontokoi176, increases in the concentration of several minerals were observed (i.e. 43.4% for copper, 33.4% for potassium, 20.4% for magnesium, 11.5% for sodium, 39.1% for phosphorus, 23.6% for sulfur, 10.8% for silica, 44.2% for zinc), while the concentrations of iron and molybdenum decreased by 13.8% and 17.5% respectively. Despite no such obvious changes shown in the *Nipponbare* and *Koshihikari* mutants, there were some changes in their mineral concentrations. In all the mutants, the concentrations of copper, manganese, sodium and zinc increased at the different rates depending on the mutant. Molybdenum decreased in the most of the mutants, and its concentration
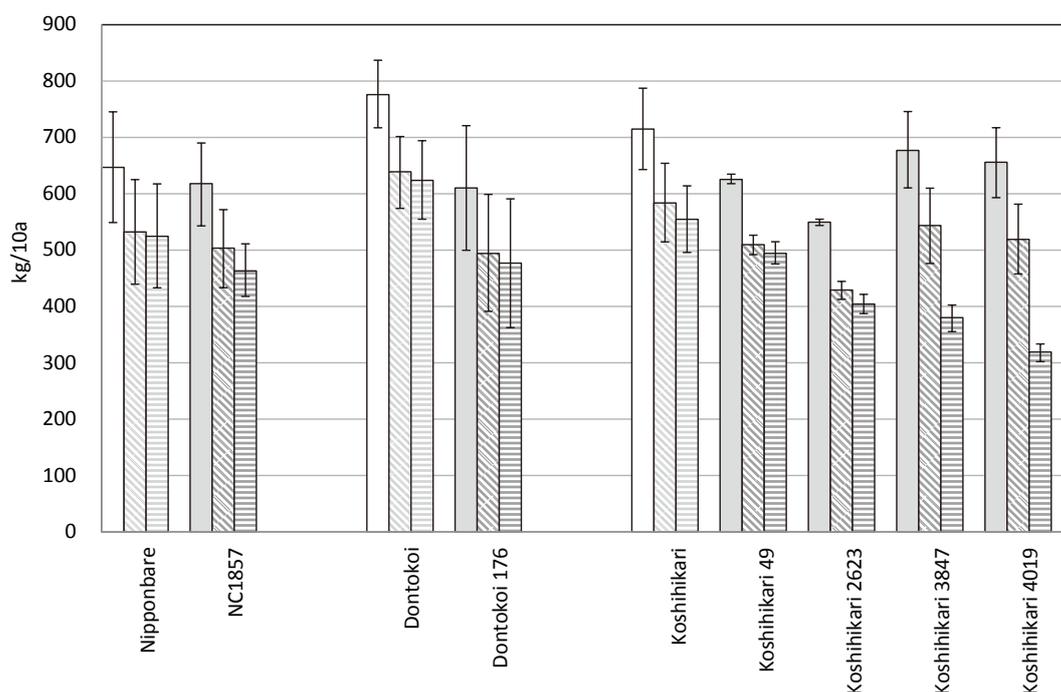
Fig. 3. Grain yield of the mutants
 Yields of dehulled grains (blank bars), gross brown grains (bars with slanted lines) and mature brown grains (bars with horizontal lines) are indicated in this order for each cultivar and mutant.
 The data are the average of observation over two years (2009 and 2010), and the error bars represent standard deviations.
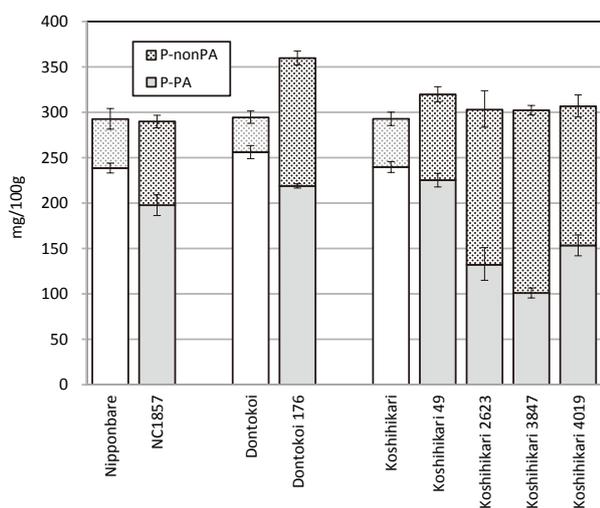

Fig. 4. Phytic acid contents of brown grains of mutants
 P-nonPA: inorganic phosphorus, P-PA: phosphorus in phytic acid
 The data are the average of observation over four years (2007 to 2010), and the error bars represent standard deviations.
 Total phosphorus and phytic acid content were analyzed, and the difference between the phosphorus values of phytic acid and total phosphorus was considered as inorganic phosphorus.

decreased 41.9% in NCI1857.

To detect the interaction between mineral ingredients and phytic acid, correlations between the mineral concentrations measured by ICP and the total phosphorus/phytic acid content analyzed by chemical procedures were calculated by pooling the all the values of the mutants and their original cultivars (Table 3). An apparent negative correlation was found between phytic acid and calcium, but the calcium content did not correlate with total phosphorus. Iron correlated positively with phytic acid content, but showed a negative correlation with the total phosphorus content. Total phosphorus correlated positively to copper, potassium, magnesium, sodium, sulfur, silica and zinc, though phytic acid content showed no correlation with any of those minerals. This result suggests the existence of some physiological or chemical interactions between phytic acid and calcium and iron in the rice endosperm.

4. Germination and seedling growth of the mutants

The original cultivars showed an approximate 100% germination rate on the first day after presoaking for two days (Fig. 5). The germination rates of Koshihikari49 and Koshihikari4019 were slightly lower on the first day, but recovered to the level of the original cultivar on the

Table 2. Mineral concentrations as measured by ICP

Minerals	B	Ca	Cu	Fe	K	Mg	Mn	Mo	Na	Ni	P	S	Si	Zn
Nipponbare	0.56	6.7	0.21	0.95	206.0	87.3	1.8	0.102	4.3	0.038	239.7	18.6	1.7	1.9
NC1857	0.53	6.9	0.22	0.88	221.2	91.8	2.4[a]	0.049[b]	4.9[a]	0.041	254.5	18.1	1.9	1.9
Dontokoi	0.55	6.5	0.22	0.96	240.9	98.8	1.9	0.080	5.2	0.031	278.9	18.8	1.9	2.2
Dontokoi176	0.55	6.7	0.31[a]	0.83[b]	321.3[a]	119.0[a]	2.0	0.066[b]	5.9[a]	0.034	387.9[a]	23.3[a]	2.1[a]	3.1[a]
Koshihikari	0.53	6.7	0.20	0.96	199.0	97.6	1.4	0.068	4.2	0.035	257.1	18.3	1.9	2.0
Koshihikari49	0.54	6.8	0.25[a]	1.00	228.3[a]	105.9	2.0[a]	0.060[b]	5.0[a]	0.036	292.0[a]	17.0	1.9	2.2[a]
Koshihikari2623	0.55	6.8	0.24[a]	0.91	227.5[a]	105.6	1.8[a]	0.069	5.0[a]	0.038	275.4	19.5	2.0	2.4[a]
Koshihikari3847	0.54	7.5[a]	0.21	0.86[b]	179.4[b]	93.3	2.0[a]	0.059[b]	4.1	0.033	245.8	18.0	1.9	2.1
Koshihikari4019	0.54	7.0	0.21	0.91	182.4[b]	95.3	1.9[a]	0.064	4.4	0.032[b]	256.3	20.2	1.9	2.0

Values are denoted in unit of mg in 100 g air-dried brown grains.
a: Increase in mutant, b: Decrease in mutant.

Table 3. Correlation between mineral contents and total phosphorus or phytic acid content

Reference	B	Ca[3]	Cu[1]	Fe[2]	K[1]	Mg[1]	Mn
Total P	0.420	-0.119	0.942	-0.463	0.783	0.873	0.167
Phytic acid	0.231	-0.806	0.122	0.544	0.394	0.092	-0.177

Reference	Mo	Na[1]	Ni	P[1]	S[1]	Si[1]	Zn[1]
Total P	-0.184	0.741	-0.248	0.934	0.745	0.757	0.911
Phytic acid	0.424	0.307	0.024	0.233	-0.017	-0.347	-0.002

1) Correlated to total P, but not correlated to phytic acid
2) Correlated to phytic acid, but negatively correlated to total P
3) Negatively correlated only to phytic acid
The correlations were calculated by the REML method.

third day. The germination rates of Koshihikari2623 and Koshihikari3847 were considerably lower than that of the original cultivar (61.7% and 40.6%, respectively) on the first day, but recovered in part as the germination treatment proceeded (70.1% and 75.3% respectively) on the third day.

Seedling growth was observed for 10 days after germination (Fig. 6). Most of the mutants showed similar growth patterns with respect to their original cultivars, even though some minor differences were observed. However, these differences in seedling growth were not significant enough to be attributable to seedling weakness observed in the seedling nursery.

Discussion

1. Mutant selection and their characteristics

The lpa mutations were not rare mutations after mutagenic treatments, but only a limited number of mutants were homo-non-lethal. In our attempt to find mutants with economical value, we successfully obtained six non-lethal distinctive lpa mutants from the screening after mutagenic treatments. The six mutants had varying degrees of reduction in phytic acid content (ranging from 42.1% to 94.1%) in comparison to the original cultivars. In contrast, the amount of inorganic phosphorus increased. The total amounts of phosphorus in phytic acid and inorganic phosphorus were almost same as that of their original cultivars except for a mutant, Dontokoi176.

In previous reports on rice phytic acid mutants, the phytic acid content of non-lethal rice mutants was shown to be around 50% to 70% of that of their original cultivars, with 25% being among the lowest reported (Kim et al. 2008). The phytic acid content of one mutant obtained in our study was also close to that low value, and the reduction

in the other mutants ranged from moderate to low, and their agronomic traits were similar to those of their original cultivars. The lowest phytic acid content levels reported in non-lethal mutants without major defectiveness were 35% in maize (Ertl et al. 1998) and 33.4% in soybean (Yuan et al. 2007) of that of the original cultivars. Empirically, the
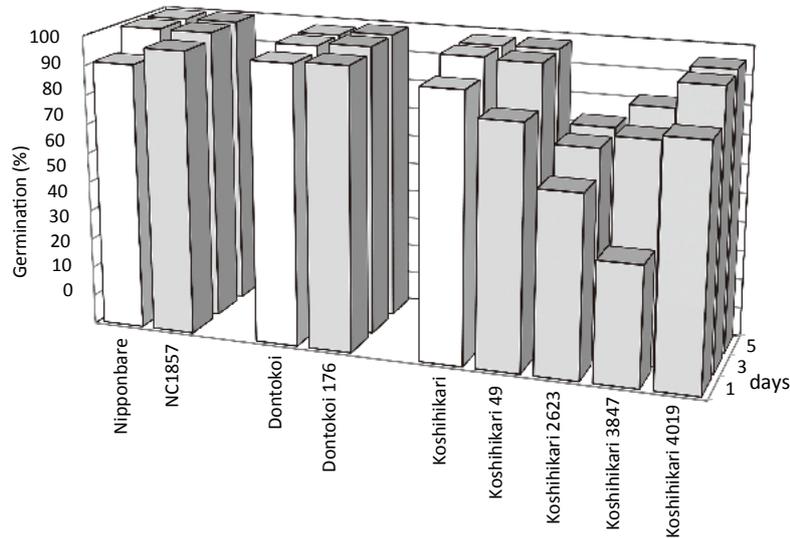

Fig. 5. Germination rate of the mutants
X axis: mutants, Y: Germination (%), Z: Days after germination treatment.
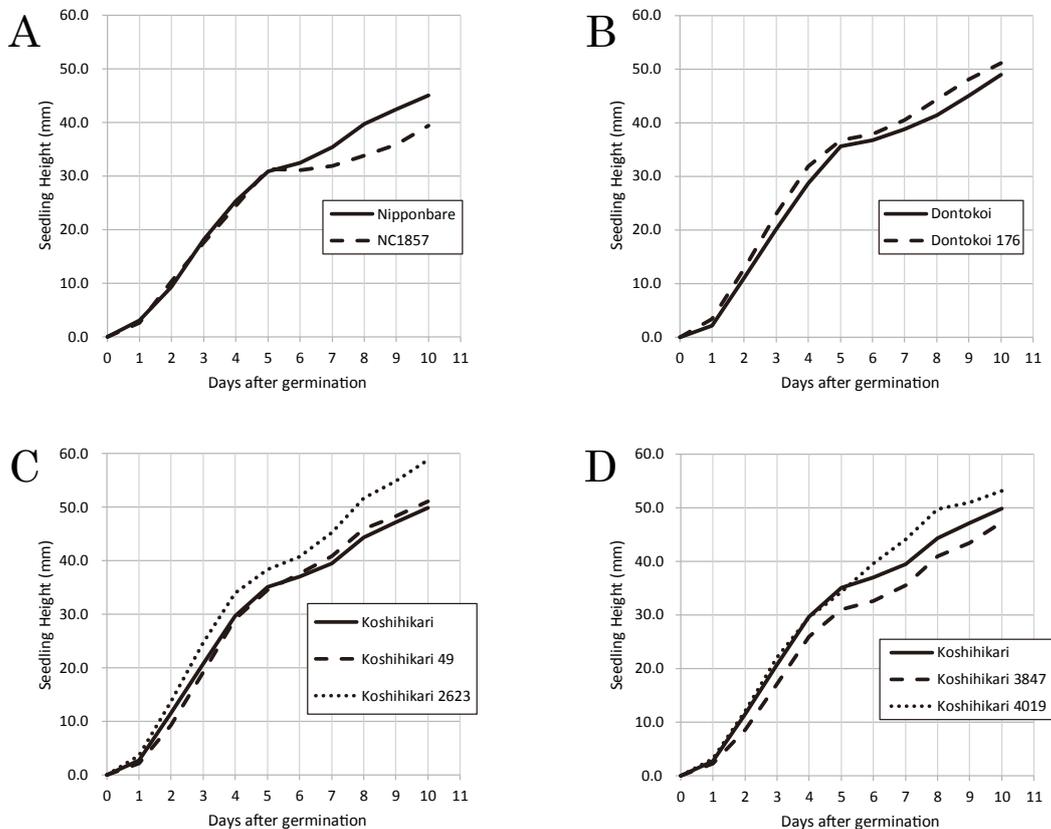Blank bars indicate the original cultivars; shaded bars indicate the mutants.

Fig. 6. Seedling growth of the mutants measured on the first ten days after germination.

phytic acid content of around 30% of that of the original cultivar might be the lowest among non-lethal mutants with healthy growth over crop species, except for the barley mutant, whose phytic acid content was 10% of that of its original cultivar (Dorsch et al. 2003).

Though our mutants showed varying degrees of weakness in their seedling stage, the field performance of our mutant lines was almost indistinguishable from their original cultivars. Among the lpa mutants that have been reported so far, our evaluations suggested that our mutants have the best possibility of becoming viable candidates of economic value with their favorable grain production and one of the lowest levels of phytic acid content.

2. The germination problem of the mutants

We have observed some signs of weakness, however, at the seedling stage in our mutants. Precise observation of germination and seedling growth after the germination showed that four of the six mutant lines exhibited germination comparable to that of their original cultivars, which showed nearly 100% germination, while the remaining two (Koshihikari2623 and 3847) showed delayed germination and slightly decreased germination rates. As these two mutant lines are among those with a lower phytic acid content among our mutants, the lpa phenotype may have a connection with lower seed germination. However, the germination of Koshihikari4019 (i.e. the second lowest lpa mutant among our mutants) showed almost the same rate as in the original cultivar, suggesting that factors other than lpa might be involved in the germination problem of our mutants, and that genetic improvement could be expected in two mutants (Koshihikari2623 and 3847). Few observations have been reported on the relationship between the lpa phenotype and decreased germination. We observed seedling growth after germination. Observing the germinated seedlings over the course of development revealed almost comparable seedling growth relative to their original cultivars, even in mutant lines with delayed germination and lower germination rates.

3. Mineral composition

In the endosperm, some of these minerals, such as iron and magnesium, have been suggested to co-localize with phytic acid in the peripheral region of grains, so as to exist as a chelating complex in the aleurone layer of the endosperm (Iwai et al. 2012). However, in most of the research reported so far, a change in mineral content had been detected with increases in iron content (Liu et al. 2007), zinc content (Liu et al. 2004), and calcium, potassium, magnesium, iron and zinc content (Ren 2007). Our results showed that phytic acid correlated positively with iron and correlated negatively with calcium. This suggested that phytic acid possibly forms a chelating complex with iron in the rice endosperm, and it is possibly replaced by calcium in the aleurone layer.

4. Nutritional value and feeding

The induction of lpa mutants in crops was introduced by Ertl et al. (1998) to improve the nutritional value of crops, by increasing the phosphorus available to non-ruminant animals and to controlling environmental pollution by reducing the residual phosphorus in animal feces. Since then, the evaluations have been repeated with various modifications and the nutritional value of lpa crop mutants had been evaluated in broiler chicks (Li et al. 2000, Yan et al. 2000, Jang et al. 2003), in chicken (Yan et al. 2000, Li et al. 2001a, Li et al 2001b, Tarkalson and Mikkelsen 2003), in pig (Veum et al. 2001, Veum et al. 2002, Thacker et al. 2004, Htoo et al. 2007, Hill 2009) and in rainbow trout (Overeturf et al. 2003), and then summarized by Raboy (2002). These evaluations have led to the conclusion that feeding crops with low phytic acid content is analogous to phytase-treated crops in terms of bone strength, promoting growth and reducing phosphorus excretion.

In our research, the decrease of phytic acid leads to the decrease of iron. Most of the iron content in grains is not nutritionally available to non-ruminant animals that include humans because the majority of iron are fixed in phytic acid molecules. Therefore, the decrease of phytic acid is expected to increase bio-available iron whose deficiency is not rare in domestic animals and humans. Moreover, the increased calcium in lpa mutants as shown in our research could contribute to improve animal and human nutrition.

Raboy (2009) also discussed the possibility of modifying the metabolism of phytic acid, thus raising the possibility of obtaining more genetic resources such as modified total phosphorus content.

In this study, we obtained six non-lethal lpa rice mutants from leading cultivars in Japan, making them cultivars with possibly with the lowest phytic acid content and with the least deleterious effect. The increased availability of phosphorus and bio-availability of iron were the major modifications made in the mutants. We believe that our mutants could be good genetic resources for improving the nutritional value of rice, both as forage rice and as a staple food for human consumption.

We employed two methods of identifying the phytic acid contents in rice grains. For mutant screening and single gain genetic analysis, a modified molybdenum blue method was used based on the assumption that inorganic phosphorus may increase in low phytic acid mutants. Conversely, accurate phytic acid content was determined by HPLC. Though the molybdenum blue method is not the direct method of determination for phytic acid, there could be a wide range of applicable situations for this quick and easy method, such as cultivar breeding and grain quality control. In addition, the molybdenum blue method may also be used

to find new mutants with increased inorganic phosphorus of a wild type level or only a slightly decreased level of phytic acid, such as Dontokoi176.

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