Plant Science 238 (2015) 170–177

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

A decrease in phytic acid content substantially affects the distribution of mineral elements within rice seeds

Hiroaki Sakai[a], Toru Iwai[b], Chie Matsubara[b], Yuto Usui[b], Masaki Okamura[b], Osamu Yatou[c], Yasuko Terada[d], Naohiro Aoki[b], Sho Nishida[b], Kaoru T. Yoshida[b,*]

[a] Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo, Japan
[b] Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan
[c] Crop Development Division, NARO Agricultural Research Center, Inada, Joetsu, Niigata, Japan
[d] Super Photon Ring-8, Japan Synchrotron Radiation Research Institute, Sayo-cho, Sayo-gun, Hyogo, Japan

ARTICLE INFO

Article history:
Received 1 April 2015
Received in revised form 30 May 2015
Accepted 6 June 2015
Available online 14 June 2015

Keywords:
Low phytic acid grains
Mineral elements
Mutant
Oryza sativa L.
Phosphorus
Seed storage substances

ABSTRACT

Phytic acid (*myo*-inositol hexa*kis*phosphate; $InsP_6$) is the storage compound of phosphorus and many mineral elements in seeds. To determine the role of $InsP_6$ in the accumulation and distribution of mineral elements in seeds, we performed fine mappings of mineral elements through synchrotron-based X-ray microfluorescence analysis using developing seeds from two independent low phytic acid (*lpa*) mutants of rice (*Oryza sativa* L.). The reduced $InsP_6$ in *lpa* seeds did not affect the translocation of mineral elements from vegetative organs into seeds, because the total amounts of phosphorus and the other mineral elements in *lpa* seeds were identical to those in the wild type (WT). However, the reduced $InsP_6$ caused large changes in mineral localization within *lpa* seeds. Phosphorus and potassium in the aleurone layer of *lpa* greatly decreased and diffused into the endosperm. Zinc and copper, which were broadly distributed from the aleurone layer to the inner endosperm in the WT, were localized in the narrower space around the aleurone layer in *lpa* mutants. We also confirmed that similar distribution changes occurred in transgenic rice with the lpa phenotype. Using these results, we discussed the role of $InsP_6$ in the dynamic accumulation and distribution patterns of mineral elements during seed development.

1. Introduction

Phytic acid, *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate ($InsP_6$), is a hexaphosphoric ester of *myo*-inositol. $InsP_6$ serves as a phosphorus (P) storage substance in plants. In plant seeds, about 75 ± 10% of total P is accumulated as $InsP_6$ [1], and $InsP_6$ is deposited primarily as mixed salts of various mineral cations such as magnesium (Mg), potassium (K), calcium (Ca), manganese (Mn), iron (Fe), and zinc (Zn) [2,3] because $InsP_6$ has six negatively charged phosphate groups and can chelate strongly with the cations to form insoluble salts; i.e., phytate.

Cereal grains are utilized not only as staple food items for humans but also as feeds for livestock. However, P in the form of $InsP_6$ is not readily available to monogastric non-ruminant animals, such as humans, pigs, and poultry, because they lack phytase, the enzyme that degrades $InsP_6$ [4]. To provide the optimal level of P for animal growth, feeds have traditionally been supplemented with inorganic phosphate (Pi) [5]. Consequently, non-ruminants excrete large amounts of unabsorbed P derived from $InsP_6$ and surplus Pi in their manure. This leads to the accumulation of P in soil and water, and subsequently to the eutrophication of water areas [5,6]. In addition, non-ruminants do not easily absorb metal elements from seeds because they bind to $InsP_6$ and form phytate [7]. To decrease the environmental loading and to improve the utilization of P and mineral cations, the development of cultivars that contain low levels of phytate in their seeds is a major breeding objective [5]. Several *low phytic acid* (*lpa*) mutants, whose seed phytate is reduced by 50–95%, have been isolated in barley [8,9], rice [10,11], wheat [12], maize [13–15], and soybean [16]. For transgenic plants, Shi et al. [17] succeeded in producing transgenic plants with a low-phytate and high-Pi phenotype in maize and soybean through the gene silencing of $InsP_6$ transporter. Kuwano et al. also generated

Abbreviations: DAF, days after flowering; EMS, ethyl methanesulfonate; ICP–OES, inductively coupled plasma optical–emission spectrometry; $InsP_6$, *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate; lpa, low phytic acid; μ-XRF, micro X-ray fluorescence; NT, non-transformant; P, phosphorus; Pi, inorganic phosphate; WT, wild type.

* Corresponding author. Tel.: +81 3 5841 8086; fax: +81 3 5841 1306.
E-mail address: ayosida@mail.ecc.u-tokyo.ac.jp (K.T. Yoshida).

http://dx.doi.org/10.1016/j.plantsci.2015.06.006
0168-9452/

transgenic rice, in which seed phytate was reduced by 68% and freely available Pi was concomitantly increased through the antisense repression of *1D-myo-inositol 3-phosphate synthase* gene [18], which catalyzes the first step in phytic acid biosynthesis and inositol metabolism. Nutrition studies of livestock and human beings have confirmed that low-phytate seeds improve the availability of both P and metals such as Fe and Ca, and reduce P excretion in manure [19,20].

However, the distribution of these metal elements in low-phytate seed is not fully understood. To compare the content and distribution of metal elements in seeds between *lpa* and wild-type (WT) plants, inductively coupled plasma optical–emission spectrometry (ICP–OES) analyses were conducted using milled rice [21,22]. Although no marked effects on the amount of metal cations were observed, there was a trend for the concentrations of several cations to increase (e.g., K and Mg) in milled *lpa* seeds compared with milled WT seeds. These results suggest that the *lpa* phenotype affects the distribution of mineral cations in a seed. However, the precise locations of elements are not clear in studies that used milled grains. Synchrotron-based micro X-ray fluorescence (μ-XRF) imaging at the Super Photon Ring-8 (SPring-8) facility is non-destructive and can provide elemental mapping images with high resolution; i.e., subcellular localizations of elements, as shown in our previous study [23]. Using μ-XRF imaging analysis, we have strongly suggested that not all storage mineral cations in a rice seed existed as phytate, and that Zn and copper (Cu) accumulated as a storage form other than phytate at least in the endosperm, because a significant amount of Zn and Cu did not colocalize with P in the endosperm tissues [23]. In contrast, K, Ca, and Fe were colocalized with P, and might primarily accumulate as phytate in the aleurone layer throughout seed development. In this study, we conducted μ-XRF analysis on the cross sections of rice seeds of the WT, two independent *lpa* mutants, and one lpa transformant, to determine the differences in the dynamic changes in the distribution of minerals between WT and low-phytate seeds, and to estimate the role of $InsP_6$ in the metal distribution. The nutritional value of the low-phytate seed in relation to the distribution pattern and the storage form of the elements was then considered.

2. Materials and methods

2.1. Plant materials

We identified two *lpa* mutants from an M_2 population of rice (*Oryza sativa* L. var *japonica*, cv. Koshihikari) mutagenized with ethyl methanesulfonate (EMS), the seeds of which had a high Pi content, because a high Pi content is an indicator of low $InsP_6$ content [5]. Self-pollinated M_3 seeds of each putative mutant were planted to confirm the *lpa* phenotype in the subsequent generation. The two mutants were crossed with indica rice (cv. Kasalath) for rough mapping. The F_2 populations segregated into the wild and *lpa* types at a ratio of 3:1, indicating that each mutant phenotype is caused by a single recessive mutation. Genomic DNA was isolated individually from these mutants and analyzed using PCR-based genetic markers to identify the molecular markers linked to the *lpa* phenotypes. The primers used for the molecular markers were as follows: a derived cleaved amplified polymorphic sequence (dCAPS) corresponded to marker 3-1, 5′-TCTAATACCATTTTTCAACGAAGC-3′ and 5′-TAATCAAGTTATTTGCTGCG-3′; the other markers are shown at http://rgp.dna.affrc.go.jp/E/publicdata/caps/index.html. *LPA2623* was mapped roughly to the 5.8 cM region located between the C63223 and C60710 markers on the long arm of chromosome 2 and *LPA3847* to the 2.4 cM region located between the 3-1 and R2247 markers on the short arm of chromosome 3 (Fig. S1). An allelism test also revealed that the loci of the two mutants, designated *lpa2623* and *lpa3847*, were distinct from each other. *OsPGK1* and *OsMRP13/OsABCC13* genes, which were responsible for low-phytate phenotype [24,25], were located in the mapped *LPA2623* and *LPA3847* regions, respectively (Fig. S1).

The mutant and WT plants were grown in pots in a greenhouse under natural light conditions in summer. Only superior caryopses were used for all experiments, because the growth of inferior caryopses was retarded [26].

To produce transgenic lpa rice, a gene consisting of 18-kDa oleosin promoter and rice *myo-inositol 3-phosphate synthase* (*RINO1*) [27] cDNA in the antisense orientation has been introduced into japonica rice (cv. Kitaake). The detailed methods of transformation, selection of a stable transformant, and growth conditions were described previously [18].

2.2. Seed Pi measurement

To analyze Pi levels, crushed powdery whole seed was extracted with 12.5% (w/v) trichloroacetic acid containing 25 mM $MgCl_2$. The Pi concentration was colorimetrically determined as described by Chen et al. [28]. The detailed method was described previously [29].

2.3. Ion chromatography analysis

Mature rice seeds were dehusked, and dried for 48 h at 60 °C. The dried seed was weighed and crushed with a hammer. The crushed samples were ground with a mortar and pestle and then homogenized in 2.4% (w/v) HCl. The HCl extracts were diluted 40-fold with deionized water and then subjected to ion chromatography. The details of the extraction and ion chromatography method were described previously [29]. More than three independent seeds were used in the experiment.

2.4. Quantitative analysis of starch

Seed starch contents were measured according to the method of Okamura et al. [30]. Two to three dehusked mature seeds were dried for 48 h at 60 °C and then ground into powders using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). The powdered samples were extracted twice with 80% (v/v) ethanol for 20 min at 80 °C, with mixing every 5 min. After centrifugation at 11,000 × *g* for 5 min, the precipitates were resuspended in distilled water, and boiled for 2 h with mixing every 30 min. The enzymatic method using an F-kit for starch (J.K. International, Tokyo, Japan) was used for the assay. The starch concentration was determined spectrophotometrically at 340 nm. The analysis was repeated at least three times using seeds derived from independent plants.

2.5. ICP–OES analysis

Mature seeds were dehusked and dried for 48 h at 60 °C. Six dried seeds were weighed and crushed together using a Multi-Beads Shocker (Yasui Kikai), and then digested with 2 mL of 0.08 N HNO_3 for 30 min at 80 °C. Subsequently, samples were heated to 120 °C for at least 1 h to evaporate water. Then, 1 mL of H_2O_2 was added and the samples were heated sequentially for 30 min at 80 °C, 1 h at 120 °C, and overnight at 80 °C. The samples were then dissolved in 1 mL of 0.08 N HNO_3. Elemental concentrations in the acid-digested samples were analyzed by inductively coupled plasma optical-emission spectrometry (ICP–OES; model SPS3500, Hitachi High-Tech Science Co., Tokyo, Japan) in accordance with the manufacturer's instructions. The experiment was repeated three times using three independent seed samples.