Is it true aleurone in the thick aleurone rice mutant?

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Abstract

Background Diet-related non communicable diseases (NCDs) impose a heavy burden on human health worldwide. Rice is a good target for diet-related disease prevention strategies because it is widely consumed. Liu et al. demonstrated that increasing the number of cell layers and thickness of putative aleurone in ta2-1 (thick aleurone 2-1) mutant rice enhances simultaneously the content of multiple micronutrients. However, the increases of aleurone-associated nutrients were not proportional to the increases in the aleurone thickness.

Results In this study, first, cytohistological analyses and transmission electron microscopy demonstrated that the multilayer in ta2-1 exhibited aleurone cell structural features. Second, we detected an increase in insoluble fibre and insoluble bound-phenolic compounds, a shift in aleurone-specific NNSP (neutral non-starch polysaccharide) profile, enhancement of phytate and minerals such as iron, zinc, potassium, magnesium, sulphur, and manganese, enrichment of triacylglycerol and phosphatidylcholine but slight reduction in free fatty acid, and an increase in oleic fatty acid composition.

Conclusion These findings support our hypothesis that the expanded aleurone-like layers in ta2-1 maintained the distinctive aleurone features and composition associated with true aleurone. We provide perspectives to achieve even greater filling of this expanded micronutrient sink to alleviate the burden of NCDs.

Background

What we know?

Globally, diet-related noncommunicable diseases (NCDs) have significant negative impacts on human health and create an ever-growing burden on health systems. According to the World Health Organization in 2014, chronic diseases such as cardiovascular diseases, cancers, and type II diabetes caused more than 63% of total deaths worldwide (Armstrong *et al.*, 2014; GBD Risk Factors Collaborators, 2016). Recognizing the compelling threat posed by NCDs, diet-related prevention strategies have become a top priority for all stakeholders with evident social and economic advantages compared to efforts to cure these conditions (Lachat *et al.*, 2013; World Health Organization, 2005). The two central concepts behind the work presented here are that: (1) health outcomes can be achieved more readily by improving the composition of staple food; and (2) it is possible to co-ordinately improve multiple nutritional factors in order to achieve desired outcomes.

Rice represents an ideal target for such compositional intervention, being a widely consumed staple in both developing and developed countries. Among the top five most populated countries, China, India, and Indonesia are the developing countries that rely heavily on rice as a staple food (FAOSTAT, 2017). White rice is the most commonly consumed type and a good source of dietary energy but poor in many essential nutrients including dietary fibre, antioxidants, phenolic compounds, minerals, and vitamins. Improving the content of any one of these alone would likely be insufficient to reduce the risk of any

specific type of chronic disease (Borresen & Ryan, 2014; Henderson *et al.*, 2012; Kahlon, 2009; Nagendra Prasad *et al.*, 2011). On the other hand, national campaigns have been initiated to promote brown rice (wholegrain rice) consumption. In the Philippines, a campaign called “BeRICEponsible” was initiated in 2014 to promote the health benefits associated with brown rice and to encourage brown rice consumption (Mohan *et al.*, 2017). In comparison with white rice, brown rice is a good source of many essential nutrients because of the presence of aleurone in bran (mixed tissues of pericarp, testa, aleurone, and embryo).

In rice, the bran tissues provide about 40% of the total minerals, 90% of the total phosphorus, 50% of the total niacin (Vitamin B3), 50% of total lipid, and about 50% of the total phenolic compounds of the whole rice caryopsis (Goufo & Trindade, 2014; Kik & Williams, 1945; Resurreccion *et al.*, 1979). Along with the embryo, aleurone is the major contributor to the micronutrients of bran, and the aleurone represents more than 66% of rice bran fraction (Taira, 1995). Aleurone is the outermost cell layer of rice endosperm and very distinctive from the inner starchy endosperm (Becraft, 2001; Wu *et al.*, 2016). In rice, the number of aleurone layers can influence the quantities of important micronutrients such as iron and zinc (Sellappan *et al.*, 2009; Yu *et al.*, 2014). In our previous work we isolated and characterised in molecular genetic detail a rice mutant *ta2-1* with thicker aleurone phenotype; a point mutation in the 14th intron of the DNA demethylase *OsROS1* led to alternative splicing responsible for the new phenotype (Liu *et al.*, 2018). In comparison with single-cell layer aleurone in the wild type, *ta2-1* has an average of 4.8 aleurone cell layers and up to 10 cell layers in some regions of the grain. Increases in nutritional components such as protein, lipid, starch, minerals, dietary fibre, antioxidants, and different vitamins were detected (Liu *et al.*, 2018). However, the increases for most of the aleurone-associated nutrients and phytochemicals were not proportional to the increases in aleurone thickness. This was particularly evident for the important minerals of iron and zinc. This may suggest that the observed extra aleurone cells may not express the full biosynthetic functionality of aleurone cells, and/or the full allocation functionality of components such as iron and zinc. In the case of minerals, the uptake, transport and/or loading capacity may affect their final contents in aleurone.

What we don’t know?

In this study, our aim is to test whether the multilayer aleurone in *ta2-1* maintains the various expected and normal aleurone identities, and acts as a source for multiple nutritional enhancements. Two approaches were developed for this aim. First, different microscopic techniques were used to visualize the spatial and temporal distribution of different cellular and subcellular elements of aleurone cells in *ta2-1* during caryopsis development. Second, additional nutritional and compositional analyses were conducted to assess whether different nutritional elements such as dietary fibre and phenolic compounds, fatty acids, minerals, and phytate in the multi-layer aleurone of *ta2-1* follow the same aleurone-specific distribution patterns as in the wild-type. The interaction of minerals and phytate and their potential effect on human nutrition will be discussed. The aim of this study is to further improve the biofortification strategy in the *ta2-1* rice background and pave the way for similar improvements in other cereals.

Materials And Methods

Statistical methods and assumptions

Tukey's Honestly Significant Difference (Tukey's HSD) test was used to test the hypothesis that the nutrient content of *ta2-1* mutant rice was different from wild-type with the null hypothesis as no difference (Kirk, 1968; Tukey, 1953). Statistical analyses were conducted using R and all the results reported as significant were those with significance level of $P<0.05$ (R Core Team, 2013). The exact p-value of all the statistical analyses in this study can be found in the file of Fig. 5-12_RawData.xlsx in the supplementary information.

Cultivation of the wild-type and ta2-1 mutant rice for analysis

Rice mutant *ta2-1* was backcrossed three times (BC3) to remove unwanted ethyl methanesulfonate mutations, following by two rounds of self-pollination and selectionto identify lines homozygous and stable for the thick-aleurone phenotype. The rice grains of BC3F3 homozygous *ta2-1* and wild-type ZH11 (Zhonghua 11) were sown side by side in the field for the grain increase in Haidian, Beijing, Peoples Republic of China (39.95^{o}N, 116.38^{o}E). In this study, 50 individual plants from *ta2-1* and wild-type ZH11 were harvested and analysed. The hull (palea and lemma) of the rice caryopsis was removed. No polishing was done so that the bran layer (including the rice aleurone layers) was maintained. In order to represent the mean of all the grains harvested and to mimic the industrial crop product processing, rice caryopses from 50 individual plants from *ta2-1* and wild-type were milled to wholegrain rice flour by Laboratory Mill 3100 (LM3100, Perten) in the same day and under similar temperature and humidity. This milled wholegrain rice flour sample was used for all the nutritional and biochemical analyses. All the replicates thus represented technical replicates from a single biological repeat of bulk wholegrain rice flour sample. BC3F4 rice seed of *ta2-1* and ZH11 lines has been analysed in Liu *et al.* (Liu *et al.*, 2018) and BC3F5 seed was used for the current study.

Sudan red staining of aleurone tissue

Stain solution was prepared by dissolving 1g of Sudan red IV in 50ml of polyethylene glycol solution (202398, Sigma-Aldrich), incubated at 90°C for one hour, and mixed with equal volume of 90% glycerol. After removing the fruit coat (palea and lemma) of each grain, mature rice grains were incubated in distilled water for five hours and then sectioned transversely or longitudinally using a razor blade. Sections were stained in Sudan red solution at room temperature for 24 to 72 hours, followed by counterstaining with Lugol's staining solution (32922, Sigma-Aldrich) at room temperature for 20min and observed under stereomicroscope (M80, Leica Microsystems) (Sreenivasulu *et al.*, 2010).

Light microscopic observation coupled with Calcofluor white or PAS and CBB staining

Rice grains were fixed in formalin-acetic acid-alcohol solution (60% ethanol, 5% glacial acetic acid and 2% formaldehyde), degassed for one hour, dehydrated in a series of alcohol solutions containing 70%, 80%,

95%, and 100% ethanol, infiltrated in LR white resin (14380, Electron Microscopy Sciences), and polymerized for 24 hours at 60°C. Microtome sectioning was done by Leica UC7 microtome (UC7, Leica Microsystems).

In Calcofluor white staining, sections were stained in 0.01% Calcofluor white solution (18909, Sigma-Aldrich) at room temperature for 2min and examined by light microscopy.

In PAS and CBB staining, the fixed sections on slides were incubated in preheated 0.4% periodic acid (375810, Sigma-Aldrich) at 57°C for 30min, followed by rinsing in distilled water for three times. Schiff reagent (3952016, Sigma-Aldrich) was applied and the slides were incubated at room temperature for 15min, following by rinsing three times in distilled water. The sections were then incubated in 1% Coomassie blue R-250 (20278, ThermoScientific) at room temperature for 2 min and rinsed three times with distilled water. Sections were dehydrated in a series of alcohol solutions of 30%, 50%, 60%, 75%, 85%, 95%, and 100% ethanol for 2min each, followed by clearing of each slide in 50% xylene and 100% xylene solution (534056, Sigma-Aldrich) for 2min each. The sections were then mounted on coverslips with Eukitt® quick hardening mounting medium (03989, Fluka) and observed under a light microscope.

Transmission electron microscopic observation

After removing the fruit coat (palea and lemma) of each grain, mature rice grains were transversely sectioned by a razor blade. Sectioned rice grain was fixed in 2.5% glutaraldehyde in a 0.1M sodium phosphate buffer (pH 7.0) for one hour, washed in the same buffer, and incubated at room temperature for 30min for three times. The fixed rice grain was then undergone post-fixation in 1% osmium tetraoxide at 4°C for 16 hours (overnight). After fixation, samples were washed in sodium phosphate buffer (pH 7.0) for 30min for three times, followed by dehydration in a series of alcohol solutions of 30%, 50%, 60%, 75%, 85%, 95%, and 100% ethanol for 10min each. The dehydrated samples were then infiltrated with acetone and Spurr® solution (14300, Electron Microscopy Sciences) in a series of solution with acetone to Spurr ratio of 1:0, 2:1, 1:1, 1:2, and 0:1 for four hours each, and embedded at 60°C for 16 to 24 hours. Embedded samples were sectioned, stained, and observed under transmission electron microscope (1230, JEOL), as reported by Roland and Vian (Roland & Vian, 1991).

Total mineral content estimation and minerals composition measurement

The total mineral content of samples was measured by ash assay according to AOAC Method 923.03 (AOAC International, 2016). In ash assay, about 2g of desiccated rice flour was heated at 540°C for 15 hours and the mass of ash residue was then weighed.

Minerals composition was determined by ICP-OES (inductively coupled plasma optical emission spectrometry) (Zarcinas & Cartwright, 1983; Zarcinas *et al.*, 1987). In minerals composition assay, about 0.5g of rice flour was digested using tube block digestion with 8M nitric acid at 140°C for eight hours. Zinc, iron, potassium, magnesium, phosphorus and sulphur content were then analysed using ICP-OES at

CSIRO, Urrbrae, Adelaide, South Australia, at Waite Analytical Service (University of Adelaide, Waite, South Australia, Australia).

Total phytic acid content

Determination of the phytate content of the flour samples was conducted based on the method of Harland and Oberleas (1986), as described in AOAC Method 986.11 (AOAC International, 2016; Harland & Oberleas, 1986). Briefly, 0.5 g cereal flour sample was weighed, extracted with 2.4% hydrochloric acid for one hour, and centrifuged. The supernatant was diluted and subjected to anion exchange column (500mg, 59822065, Agilent Technologies) to remove the non-phytate elements. Phytate bound to the column was then eluted with 2M hydrochloric acid. Phosphorous levels were determined by spectrophotometer using the molybdate and sulphonic acid colouring method with absorbance readings at 640nm. Phytate was calculated using the following formula:

Phytate (mg/g) = P conc*V1*V2/(1000*sample weight*0.282)

where P concentration is the concentration of phosphorous (µg/ml), as determined by spectrophotometry, V1 is the volume of the final solution, V2 is the volume of the extracted phytate solution, and 0.282 is the phosphorus to phytate conversion factor.

Fibre content

Total dietary fibre was measured according to AOAC Method 985.29 and soluble and insoluble fibre according to AOAC Method 991.43 (AOAC International, 2016).

Samples of 1g of rice flour were undergone sequential enzymatic digestions of heat stable α-amylase (300 U/ml, E-BLAAM, Megazyme) and amyloglucosidase (3300 U/ml, E-AMGDF, Megazyme) to hydrolyse the starch content, followed by protease (350 tyrosine units/ml, E-BSPRT, Megazyme) to depolymerise the protein content. After the enzymatic digestions, different extraction and purification methods have applied to yield the total-, soluble- and insoluble-fibre fractions. Direct precipitation of sample after enzymatic digestions yielded the total-fibre; filtration of the enzymatic digested sample through Celite (61790-53-2, Sigma-Aldrich)-embedded fritted glass crucible, following by ethanol precipitation of the filtrate yielded the soluble-fibre while the residue after filtration represented the insoluble-fibre. After the enzymatic digestions and fibre extraction, the total-, soluble- and insoluble-dietary fibres were determined by the gravimetric measurements of the residue dry mass of the total-precipitated-residue, the filtrate-precipitated-residue and the residue after digestion respectively, with the correction from the parallel measurement of ash and protein content of the triplicate of each sample.

Total NNSP

NNSP was measured by the gas chromatographic procedure according to AOAC Method 994.13, as detailed by Theander *et al.* with slight modification (AOAC International, 2016; Theander *et al.*, 1995). Briefly, the total-dietary fibres extracted from total dietary fibre assay were hydrolysed with sulfuric acid.

The neutral sugars released were then reduced by potassium borohydride solution and acetylated by acetic acids to alditol acetates, which are then quantified by gas chromatography. Gas chromatography conditions: BPX70 column (30m x 320µm x 0.25µm); injection 0.5µl; inlet at 240°C; 20:1 split; 1.62ml/min constant flow; oven at 180°C for 2min, 12^{o}C/min to 230°C and hold for 8min; flame ionization detector at 250°C; quantification against internal allose standard.

Total phenolic compounds

Total phenols assay by FCR is an electron transfer-based assay, which estimates the amount of antioxidant based on the reducing power (willingness to donate electrons) of the antioxidants against oxidant probe molybdates, quantifies the reducing power with gallic acid standards, and presents as the value of gallic acid equivalents. Combined with the differential extractions of the free-, conjugated-, bound-, and total-fractions, FCR assay is good at determining the antioxidant capacity of different groups of phenolic compounds.

Total phenolic compounds content, as well as phenolic compounds in the free-, conjugated-, and bound-states were determined according to the method described by Li *et al.* with minor modifications (Li *et al*., 2008). Briefly, these four types of phenolic compounds were extracted from 100mg samples with different extraction methods. Total-phenolic compounds were determined using 100mg of samples, adding 200µl 80% methanol, followed by alkaline hydrolysis with (2M) sodium hydroxide; free-phenolic compounds were represented by the 80% methanol extraction of the 100mg samples; conjugated-phenolic compounds were the alkaline hydrolysed products from the 80% methanol extract; bound-phenolic compounds were the alkaline hydrolysed products from the residues followed by the methanolic extraction of the free-phenolic compounds.

The amount of phenolic compounds in the treated/extracted samples was measured using FCR assay with reference to standard curve of known gallic acid concentrations. 1ml of standards were added to 4ml glass tubes. For test samples, 100µl aliquots of thoroughly mixed samples were added to 900µl water in 4ml glass tubes. 100ml of FCR (F9252, Sigma-Aldrich) was then added to each tube and vortexed immediately. 700µl sodium bicarbonate solution (1M) was added after 2min and mixed by vertexing. Each solution was incubated at room temperature in the dark for one hour. Absorbance was read at 765nm. Results were expressed in µg gallic acid equivalents/g sample.

Folate content

Total folate (Vitamin B9) was measured by commercially available VitaFast Folic acid kit (Folic Acid AOAC-RI, 100903, R-Biopharm) according to AOAC method 2004.05 as described by DeVries *et al.* (DeVries *et al*., 2005). The method incorporated the *in vitro* enzymes digestion and the growth response of the *Lactobacillus* to folate concentration in culture medium. First, pancreatin digestion was used for the release of the food matrix-bound folic acid. About 1g of rice flour and 20mg pancreatin were added and filled up to 40ml with phosphate buffer (0.05M, 0.1% ascorbate, pH7.2), and the sample was incubated at 37°C for two hours in dark. These allowed the digestion of food matrix by protease and

amylase in pancreatin and the hydrolyzation of polyglutamates to diglutamates by pancreatic conjugase to release food-bound folate which can be metabolized by *Lactobacillus*. After the incubation, the 1.5ml reaction was centrifuged at 8000 x g for 5min, diluted to 10ml with sterile distilled water, heated at 95°C for 5min, and filtered through a 0.2μm filter to get rid of the insoluble or undigested suspensions in the sample. The filtrate was then diluted in several dilutions, added into the wells of a microtiter plate coated with *Lactobacillus casei* subspecies *rhamnosus*, and incubated at 37°C for 44 to 48 hours. After the incubation, the OD at 610-630nm of the sample was measured and compared with the folate calibration standard. As the basal culture medium is lack of folate, the growth of the coated *Lactobacillus*, represented by the increase in turbidity of bacterial culture, is positively correlated with the extracted folate content in the sample. The folate concentration of the sample can be measured by comparing difference between the absorbance of the test sample with the calibration standards at OD 610-630nm.

Total lipid content

Total lipid was measured according to AOAC Method 983.23 (AOAC International, 2016). Sample of 5g rice flour was incubated with 1% Clarase (MC23.31, Southern Biological) in 0.5M sodium acetate solution at 45°C for one hour. Lipids were extracted from the sample into chloroform/methanol by multiple extractions. The samples were then subjected to homogenization with multiple additions of chloroform/methanol. After centrifugation of the mixture into separate phases, the chloroform/methanol fraction was removed and dried at 101°C for 30min to recover the lipid. The total lipid in the sample was represented by the mass of residue after drying.

TAG, FFA, and PC fractionation and quantification

According to the methodologies described by Liu *et al.*, the extraction of total lipid, the fractionation of neutral lipid (mainly TAG), free fatty acid, and polar lipid (mainly PC), and the quantification of lipid were carried out (Liu *et al.*, 2017). Thin layer chromatography (TLC) was first carried out to separate the neutral lipid and free fatty acid in solvent matrix of hexane:diethyl ether:acetic acid in 70:30:1 volume ratio. Then, another TLC was conducted in solvent matrix of chloroform:methanol:acetic acid:distilled water in 90:15:10:3 to separate the polar lipid PC. After TLC, samples were collected and lipid content in the fraction was extracted. The lipid fractions were undergone fatty acid methyl esters (FAME) and gas chromatography (GC) analyses, and quantified according to the methods described by Vanhercke et al. (Vanhercke *et al.*, 2014).

Total β-glucan

Rice β-glucan was measured with reference to the methods in AOAC Method 995.16 (AOAC International, 2016).

Briefly, 20mg of rice flour were subjected to sequential enzymatic digestions of Lichenase and β-glucosidase, followed by quantification of the released glucose with standard glucose oxidase/peroxidase (GOPOD) system. Firstly, 200μl 50% ethanol was added into 20mg of rice flour, then

1ml sodium phosphate buffer (20mM, pH6.5) was added and incubated at 100°C boiling water for 3min. Secondly, the reaction was briefly cooled and diluted. Thirdly, 10µl Lichenase (1U/µl) was added and incubated at 40°C for 1hour. The sample was then diluted by adding 3.8ml distilled water followed by centrifugation. Fourthly, the β-glucosidase reaction was conducted by adding 10µl of β-glucosidase (2U/ml) into 10µl sample supernatant collected in last step and incubated at 40°C for 15min.

Quantification of the glucose through GOPOD was conducted after the enzymatic reactions. Firstly, in 20µl sample, 150µl of glucose oxidase/peroxidase (GOPOD) reagent was added and incubated at 40°C for 20min. Secondly, the absorbance was measured at 510nm for each sample (E_A) and reagent blank (E_{BLANK}). The β-glucan content was measured using the following formula:

β-glucan (%Weight/Total weight) $= \Delta E \times F \times 600 \times 1/1000 \times 100/W_d \times 162/180$

$= \Delta E / \Delta G / W_d \times 135$

in which

ΔE = the difference between E_A and E_{BLANK},

F i= the absorbance of 2.5µg of glucose, and

W_d = weight of sample analysed (in mg) times 0.86 ($W_d \times 0.86$).

Results

The time course of aleurone development in ta2-1

To compare the aleurone development in *ta2-1* and wild type, caryopses of 6, 8, 10, 12, 15, 18, 21, 24, and 30DAA (days after anthesis) were examined. These time points represent important developmental phases such as aleurone cell fate differentiation, aleurone nutrient accumulation, and aleurone maturation. These caryopses were sectioned and stained with Sudan red plus Lugol's iodine. The aleurone cells in *ta2-1* become prominent between 6 to 8DAA, being positively stained red by Sudan red (Fig. 1) indicating the dominance of lipid and almost complete absence of starch. Wild type had a thick aleurone resembling *ta2-1* up to 10DAA. From 12DAA onwards, *ta2-1* showed a thicker Sudan red staining area, although a slight decline of red staining area at 24 and 30DAA. Although there is no indication that starchy endosperm cells can become aleurone, it seems possible that some cell layers that initially show the compositional attributes of aleurone can become starchy endosperm in the later stages of development. In maize, *dek1* mutation triggered the transdifferentiation of the aleurone to starchy endosperm cells in the late stage of development (Becraft & Yi, 2011). Furthermore, this switch is more pronounced in wild type caryopses than in *ta2-1*, leaving *ta2-1* with a thicker aleurone at maturity.

General cell structure of ta2-1

To assess the cellular and subcellular identities of aleurone cells in rice *ta2-1*, light microscopy coupled with two different staining methods were adopted.

Firstly, using periodic acid-Schiff and Coomassie brilliant blue staining (PAS-CBB), multiple layers of putative aleurone cells appeared distinctly different from starchy endosperm cells at the periphery of the mature rice caryopsis in *ta2-1*. The multiple layers of putative *ta2-1* aleurone cells had thicker cell walls (stained pink by PAS) than the inner starchy endosperm cells (Fig. 2b and d, black arrow), prominent protein bodies (stained blue by CBB) (Fig. 2e, asterisk), and much lower starch granule density (Fig. 2f and g, black closed triangle). The putative aleurone cells in *ta2-1* were more irregular in shape than wild type aleurone cells, and with fewer but larger protein bodies. Moreover, their cell distribution patterns were different. In wild type, there was one aleurone layer on the ventral side (Fig. 2a) and up to five aleurone layers on the dorsal side of the rice caryopsis (Fig. 2c). In *ta2-1*, the aleurone layer was about three layers thick on the ventral side (Fig. 2b) and more than eight layers (Fig. 2d) on the dorsal side.

Secondly, Calcofluor White staining was applied with epifluorescence microscopy to study the cell wall structure specifically. Calcofluor White binds to cellulose and other β-glucans in cell wall (Hughes & McCully, 1975). In both wild type and *ta2-1*, the outermost compressed cells of the testa and the aleurone were actively stained while the innermost starchy endosperm cells were not (Fig. 3a and b). The putative extra aleurone layers of *ta2-1* resembled authentic aleurone in this distinctive cell wall trait.

The accumulation of aleurone grains in ta2-1

Transmission electron microscopy was used to examine the subcellular details of the putative aleurone cells in *ta2-1*. In both wild-type and *ta2-1* aleurone cells, thick cell walls were evident (Fig. 4a and b, between two arrows). Also, protein bodies (sometimes referred to as aleurone grains (AG)) were abundant in both genotypes. As in the light microscopy study, AG in *ta2-1* were generally larger than the wild type with similar subcellular content. The *ta2-1* AG had other more subtle differences compared to wild type AG. Surrounded by tonoplast membrane, AG accumulate protein presumably as a source of amino acids for germination; they consist of three morphologically distinct features called the crystalloid, matrix, and globoid. These three features were present with similar electron density in the aleurone grains of both the wild type aleurone and the expanded *ta2-1* cells (Fig. 4c and e, arrows), however, the *ta2-1* AG were more variable in size with many being larger.

Together, the results from light microscopy and electron microscopy supported the conclusion that the putative extra aleurone cells in *ta2-1* retained the aleurone cell features of thick cell wall, low content of starch granules and presence of protein-rich AG. Distinctive AG features of crystalloid, matrix, and globoid were conserved in *ta2-1*. However, the *ta2-1* aleurone cells had a greater variability in cell size and shape of AG.

To measure the spatial distribution of aleurone cells, light microscopy coupled with PAS was adopted. From the outer to inner layers of the *ta2-1* aleurone, there was a change in structure and subcellular content of the AG. In wild type, only one type of AG was observed. The wild-type AG had a small internal

cavity and abundant protein matrix that was stained deep blue by CBB. Moreover, individual aleurone cells were smaller in size (Fig. 2a and c) to those in *ta2-1* (Fig. 2b and d). The subcellular content and structure of the wild-type AG was uniform and constant. However, in *ta2-1*, two types of AG could be distinguished, i.e. AG with wild type morphology (Fig. 2e and f, single asterisk) and larger aleurone grains (LAG) (Fig. 2g, double asterisks). AG and LAG had similar deep-blue protein matrix feature, however, LAG had a larger internal globoid cavity with sometimes more limited protein matrix than AG. Moreover, the distribution patterns of LAG changed from outer to inner layers of the *ta2-1* aleurone. In the outer layers of *ta2-1* aleurone, the dominant storage compartment is AG while in inner layers, the dominant storage vacuoles resemble the LAG structure.

The contents of dietary fibre, phenolic compounds, and antioxidants

The aleurone cell wall in brown rice is an abundant source of dietary fibres. We previously showed that total dietary fibre content was increased in *ta2-1* (Liu *et al.*, 2018). In this study, we compared the insoluble- and soluble- dietary fibre in the total fibre content of *ta2*-1. Similar to our previous findings, rice mutant *ta2-1* showed 66% increase in total dietary fibre. This was largely due to a 55% increase in insoluble-dietary fibre, while no significant change (P=0.90>0.05) was observed in the content of soluble-dietary fibre (Fig. 5). Since it is known that the fibre associated with the aleurone cell wall is insoluble in nature (Collins *et al.*, 2010), the observed increase in total and insoluble fibre in *ta2-1* is consistent with the thickened aleurone being the source of the extra fibre.

Dietary fibre is composed of neutral non-starch polysaccharide (NNSP), nondigestible oligosaccharide, resistant starch, lignin, and uronic acid containing polysaccharides (Theander *et al.*, 1995). Among these components, NNSP is the major contributor of the total dietary fibre content in rice caryopsis (Collins *et al.*, 2010). The total NNSP content in the wholegrain flour of *ta2-1* was 61% higher than that in the wild-type (Fig. 6a). Moreover, being consistent with the earlier studies in different wholegrain (brown) rice varieties (Collins *et al.*, 2010; Demirbas, 2005), the β-glucan content in the wild type and *ta2-1* was very low at 0.63g and 0.59g per 100g respectively (Fig. 5). Aleurone is rich in arabinoxylan and low in cellulose and β-glucans, but starchy endosperm has an even distribution of these three fibre types (Fincher & Stone, 2004). The arabinose content increased from 18% in the wild type to 24% in *ta2-1*, and the xylose content from 17% to 20%; while glucose content dropped from 50% in the wild type to 40% in *ta2-1* (Fig. 6b), leading to changed NNSP composition profile in *ta2-1* (Fig. 6c). The basic monosaccharide units of arabinoxylan are arabinose and xylose, and the basic monosaccharide unit of cellulose and β-glucans is D-glucose. The increase in arabinose and xylose content at the expense of D-glucose in NNSP suggested that the expanded aleurone layers in *ta2-1* maintained the aleurone cell identity of arabinoxylan-rich cell wall and accounted for the composition change in total NNSP.

Antioxidants of rice include a wide spectrum of biomolecules such as phenolic compounds, uronic acids, tocopherols, carotenoids, ascorbic acid, and gamma-oryzanol which stabilize multiple oxidant sources and free radicals by their electron-transferring or hydrogen-transferring ability (Prior *et al.*, 2005). Some antioxidants can be esterified and bound to the aleurone cell wall. Our previous studies focused on the

electron-transferring ability of wholegrain rice flour through ORAC (oxygen radical absorbance capacity) and the hydrogen-transferring ability through FCR (Folin-Ciocalteu reagent) (Liu *et al.*, 2018). Here we tested their association with the aleurone cell wall, by extracting antioxidant fractions with different solubility and measuring their hydrogen-transferring abilities.

Total-phenolic compounds in plants may exist in soluble free, soluble conjugated (esterified), and insoluble bound forms (Robbins, 2003). The antioxidants were first extracted into the four fractions of total-, bound-, conjugated-, and free- phenolic compounds from the wholegrain rice flour samples. Their anti-oxidation abilities were then measured by the FCR assay. In both wild-type and *ta2-1*, about 70% of total phenolic compounds in rice caryopsis were insoluble bound-phenolic compounds, followed by 16% soluble free- and 10% soluble conjugated-phenolic compounds. Compared with the wild-type, *ta2-1* showed 43% increase in total-, 31% increase in insoluble bound-, 26% increase in soluble free- (not statistically significant) and 99% increase in soluble conjugated-phenolic compounds (Fig. 7). This pattern of increases is consistent with the increased aleurone thickness being responsible for the increased antioxidant capacity.

Folate (Vitamin B9) has potent antioxidant activity (Atteia *et al.*, 2009). In rice, the aleurone-enriched bran fraction is rich in folate (Houston & Kohler, 1970). In this study, compared with the wild-type, *ta2-1* had 32% increase in folate content (Fig. 8).

The contents of minerals and phytate

Analyses of phytate and phosphorus content revealed increases of 18% and 22% respectively in *ta2-1* compared to wild type (Fig. 9). Assuming the molecular mass of phytate is 660.04gmol^{-1} and that of phosphorus is 30.97gmol^{-1} and each phytate molecular consists of six phosphorus molecules, 75.10% and 72.47% of total phosphorus are bound to phytate in wild type and *ta2-1* respectively (Supplementary Table 1). These measurements resemble the finding of others in rice caryopsis that 73.7% of total phosphorus content is bound to phytate (Ravindran *et al.*, 1994). The similarity of percentage increase between phytate (18%) and phosphorus (22%) and the similar proportion of phosphorus bound to phytate suggested the increase of phosphorus in *ta2-1* is fully explained by the increase in phytate. Moreover, in addition to the increases in iron, zinc, and magnesium as observed by Liu *et al.* (2018), increases in manganese, potassium, and sulphur but not calcium content was observed (Fig. 10).

Supplementary Table 1 Phosphorus content bound to phytate in rice wholegrain samples.

Sample	No. of mole of			P in phytate / Total P content
	Phosphorus[1]	Phytate[2]	P in Phytate[3]	
ZH11	12.625X10^{-3}	1.580 X10^{-3}	9.481 X10^{-3}	75.10%
ta2-1	15.402X10^{-3}	1.860 X10^{-3}	1.116 X10^{-3}	72.47%

Calculation of phosphorus content is based on the equations as follows:

[1] The molar mass of phosphorus is $30.97 gmol^{-1}$, therefore, mole of phosphorus = mass of phosphorus / molar mass.

[2] The molecular mass of phytate is $660.04\ gmol^{-1}$, therefore, mole of phytate = mass of phytate / molecular mass.

[3] As the molecular formula of phytate is $C_6H_{18}O_{24}P_6$, one mole of phytate contains six moles of phosphorus.

The compositions and contents of lipid

It has been shown before that *ta2-1* mutant had higher total lipid content (Liu *et al.*, 2018). The total oil content and composition in rice aleurone (rice bran) is different from starchy endosperm (Choudhury & Juliano, 1980a, 1980b). These authors showed that the neutral lipids (largely triacylglycerol (TAG)) were concentrated in the bran (embryo and aleurone), while the phospholipids were equally distributed in the bran and starchy endosperm fractions. In our studies, lipid components were separated using thin-layer chromatography and fatty acid content quantified by gas chromatography. In both *ta2-1* and wild type, TAG was the dominant type of lipid, followed by FFA (free fatty acid), and PC (phosphatidylcholine). In comparison with wild type, *ta2-1* had a 79% increase in TAG (from 1.89% to 3.32%), 97% increase in PC (from 0.02390% to 0.04715%), and a 7% decrease in FFA (from 0.2171% to 0.2004%; not statistically significant) in wholegrain rice flour samples (Fig. 11). The large increase in TAG is expected if the thick aleurone is responsible for the increase in total lipid. There were changes in fatty acid profile in *ta2-1* (Fig. 12a), including 32% increase in oleic acid content (from 32% to 42% of the total fatty acid), 22% decrease in linoleic acid content (from 35% to 28%), and 5% decrease in palmitic acid content (from 17% to 16%) (Fig. 12b).

Discussion

General aleurone identity of the ta2-1 thick aleurone

The aleurone cells in *ta2-1* maintained the aleurone features of thick cell wall, with few starch grains, an abundance of AG and lipid bodies, and as a consequence increases in dietary fibre, phenolic compounds, lipid composition, and changes to fatty acid profile matching what would be expected if the increases come from normal aleurone cells.

Cytohistology with Calcofluor white staining, cytohistology with PAS-CBB staining, and transmission electron microscopy all demonstrated a dramatic expansion of thick cell walls in the entire *ta2-1* aleurone. The AG in *ta2-1* aleurone cells had the same distinctive structures of crystalloid, matrix, and globoid as wild-type AG, however, they were more variable in size with many being larger than in wild-type aleurone due mainly to larger globoid.

The increase in dietary fibre content in brown rice flours of *ta2-1* was mainly due to insoluble-dietary fibre, the type of fibre that forms aleurone cell walls (Collins *et al.*, 2010). The increase in arabinose and xylose

content *in ta2-1* flour and decrease in D-glucose was also consistent with the predominance of arabinoxylan in aleurone cell walls (Fincher & Stone, 2004).

The enhanced thick cell wall in *ta2-1* also changes the phenolic compound composition. Associated with the cell wall are the phenolic compounds such as ferulic acid and ρ-coumaric acid. They are mainly bound to NNSP (Goufo & Trindade, 2014). In *ta2-1*, the increase in total phenolic compounds was mainly attributable to an increase in insoluble bound-phenolic compounds.

Sudan red staining confirmed the enrichment of lipid bodies in the thickened *ta2-1* aleurone. The increase in TAG and PC in *ta2-1* flour but slight decrease in FFA, was consistent with a shift to aleurone- (bran-) specific lipid composition. Lipids in rice can be classified into non-starch lipids and starch-associated lipids (Zhou *et al.*, 2003). Rice aleurone (bran) contributes to about 40% (39-41%) of total non-starch lipids that mainly consist of TAG and PC, while starchy endosperm stores about 60% (48-71%) of total starch-associated lipids with FFA as one of the major components (Choudhury & Juliano, 1980a, 1980b). In *ta2-1* flour, the 79% increase in TAG, 97% increase in PC, and the 7% decrease in FFA is consistent with the thick aleurone maintaining normal aleurone cell function and composition. On the other hand, as the starchy endosperm content is reduced in *ta2-1*, the content of starch lipid FFA decreases.

The enrichment of lipid bodies in *ta2-1* aleurone also modifies the fatty acid profile. The fatty acid composition profiles of aleurone (bran) and starchy endosperm are different. Rice aleurone is rich in oleic acid (36% of total fatty acid) and linoleic (37%) but low in palmitic acid (23%) content. In starchy endosperm, linoleic and palmitic acid content are higher (41% and 33% respectively) but the oleic acid content is lower (20%) (Choudhury & Juliano, 1980a, 1980b). The 32% increase in oleic, 22% decrease in linoleic, and 5% decrease in palmitic acid proportions again indicates that the multilayer aleurone in *ta2-1* maintains the aleurone-specific fatty acid profile.

Aleurone cells are rich in mitochondria (Jones, 1969). In this study, no direct measurement of mitochondria was conducted, however, the folate content may be indicative of mitochondria abundance. Folate is enriched in aleurone-enriched bran fraction (Houston & Kohler, 1970). In plant, folate is synthesized in three subcellular compartments in which the final five steps are conducted in mitochondria (Gorelova *et al.*, 2017). About 30 – 50% of folate in cells is stored in mitochondria to help maintain the mitochondrial DNA stability (Depeint *et al.*, 2006). Therefore, the 32% increase in folate may indirectly indicate that the multilayer aleurone in *ta2-1* maintains the high mitochondrial content of true aleurone.

Together, the cell structural features, the increase in insoluble fibre and insoluble bound-phenolic compounds, shift in aleurone-specific NNSP profile, enrichment of TAG and PC but slightly reduction in FFA, and increase in oleic fatty acid composition collectively support the hypothesis that the additional aleurone-like layers in *ta2-1* maintain the distinctive features and composition of true aleurone cells. It's the expansion of the aleurone layer that results in an increase of the nutrients that are associated with true aleurone.

The improvement of multiple nutritional factors in *ta2-1* can help prevention of NCDs. For example, dietary fibre can maintain gastrointestinal health by increasing fecal bulk, decreasing transit time, decreasing the gastrointestinal contact of foodborne carcinogenic compounds, binding to mutagens, and lowering colonic pH (Glitsø *et al.*, 1998; Le Gall *et al.*, 2009). The insoluble antioxidants bound to cell wall materials can provide an antioxidant environment contributing to protection of the colon tissues from cancer (Sengupta *et al.*, 2001). Phytate has potential anti-neoplastic and antioxidant functions (Fox & Eberl, 2002; Norhaizan *et al.*, 2011). The multilayer aleurone rice with true aleurone composition can coordinately improve multiple nutritional factors to achieve diverse protective outcomes. In countries with rice-dominant diets, the consumption of wholegrain rice is very low (Cleveland *et al.*, 2000; Harnack *et al.*, 2003), but there is a growing trend from white rice to brown rice (with aleurone) consumption due to improved health consciousness and education (Selvam *et al.*, 2017). By improving the nutrient composition of brown rice that is more readily accepted by the public, the multilayer aleurone rice *ta2-1* could therefore deliver substantial public health advantage without remarkably changing the dietary habits. Furthermore, light polishing of thick aleurone rice retains more aleurone than wild type rice and therefore also retains more of the nutrients.

The commensurate increase in minerals and phytate content

Wholegrain flour of *ta2-1* had an 18% increase in phytate content. Using ICP-OES, we confirmed and extended our previous findings (Liu *et al.*, 2018), showing *ta2-1* also had 14 to 23% increased content of various minerals.

Aleurone is a concentrated source of many essential minerals. In synchrotron X-ray fluorescence microscopy imaging study of rice, iron, zinc, manganese, and copper were highly concentrated in the aleurone layer (Hansen *et al.*, 2012). Most of the minerals in rice are associated with phytate (Hansen *et al.*, 2012; Mills *et al.*, 2005; Simic *et al.*, 2009). Wholegrain rice and wheat have similar phosphorus content (337mg vs 323mg/100gDW) (USDA, 2019), however, rice has a higher proportion of phytate phosphorus than wheat, accounting for 77% of the total phosphorus content (Ravindran *et al.*, 1994). Phytate has a strong affinity for chelating minerals such as Zn, Fe, and Mg, which limits their absorption in the small intestine (Bohn *et al.*, 2007; Raboy, 2009). This raises concern that phytate may impair small intestinal mineral absorption and compromise mineral status.

Wholegrain wheat flour has higher phytate and iron content than processed white flour, and in an *in vitro Caco-2* cells test, wholegrain wheat flour led to a lower ferritin response than processed white flour, suggesting that the iron content in white flour is more biologically available for cellular absorption and assimilation to ferritin (Eagling *et al.*, 2014). Stevenson *et al.* summarized the findings in wheat and suggested that the consumption of wholegrain wheat or wheat bran decreased the calcium and zinc bioavailability (Stevenson *et al.*, 2012).

However, the *Caco-2* test may not reveal the full picture concerning bioavailability. Diets high in whole grains do not adversely affect mineral nutrition but have favorable health outcomes. Different recommendations of daily wholegrain intake have been proposed in the U.S., Canada, and Australia, and

no unfavourable health outcome has been reported regarding the high consumption of whole grains (Health Canada, 2011; HHS & USDA, 2015; National Health and Medical Research Council, 2013). Recently, a meta-analysis provide further evidence that higher wholegrain consumption is associated with reduced risk of digestive tract cancers (Zhang *et al.*, 2020). In both short-term (4 weeks) and long-term (2 years) studies in young and older women, the diet supplemented with phytate-rich wheat bran had no significant effect on different osteoporosis markers (Chen *et al.*, 2004; Zittermann *et al.*, 2007). Likewise, zinc absorption in young children was not negatively impacted by added phytate (Miller *et al.*, 2015). Moreover, there is no consensus on the effect of phytate on iron bioavailability (Stevenson *et al.*, 2012).

The digestion of phytate can happen in the human large intestine to release the chelated minerals for absorption. In pig studies, nearly complete (more than 97%) phytate digestion was observed in faecal samples following a normal diet with low intrinsic feed phytase (Schlemmer *et al.*, 2001). In these studies, the highest phytate degradation occurred in the large intestine rather than the stomach or small intestine. In human faecal studies, the dietary phytate degradation rate varied between 50 and 90% (Joung *et al.*, 2007; McCance & Widdowson, 1935; Walker *et al.*, 1948). In a human trial with both young and elderly women, it was also reported that the diet with high phytate content could enhance phytate degradation (Joung *et al.*, 2007). In the large intestine, the microbial phytases, foodborne phytases in plant food sources, and endogenous phytases can degrade cereal-grain phytate to release the minerals bound in the mineral-phytate inclusion for human absorption (Sandberg & Andlid, 2002). Moreover, the fermentation of the NNSP and different dietary fibres in rice can potentially acidify the luminal environment of the large bowel (Koh *et al.*, 2016). This can improve the mineral bioavailability. Therefore, the commensurate increments of minerals and phytate in *ta2-1* may not necessarily decrease the mineral availability; on the contrary, the increased phytate content in *ta2-1* may stimulate the microbial phytate degradation in large intestine, releasing bioavailable minerals and digested phytate for human absorption and gut microbiome development. However, more studies regarding the minerals' bioavailability should be conducted to further test this hypothesis.

Why are the nutrient increases not greater?

The increases in multiple nutrients in *ta2-1* flour are significant but not in proportion to the increased layers or volume of aleurone cells. We briefly explore here two hypotheses, which are not mutually exclusive, that might explain this "nutrient gap".

Hypothesis 1: The new larger aleurone may have a lower capacity to synthesize and/or store nutrients compared to wild-type aleurone.

While we have shown the expanded aleurone in *ta2-1* has the general features and composition of normal aleurone, there may be more subtle differences in biosynthetic or storage capacity. In rice, both wild-type and *ta2-1* aleurone cells have prominent AG. However, the AG in *ta2-1* aleurone cells are more variable in morphology and distribution pattern from outer to inner layers.

The rice *ta2-1* had two types of AG, morphologically normal AG and LAG. While both types of aleurone grains had prominent globoid, crystalloid, and matrix structures, LAGs in *ta2-1* had enlarged globoid cavities. The crystalloid is the storage compartment for the integral membrane proteins, whereas the matrix contains soluble protein (Jiang *et al.*, 2002; Jiang *et al.*, 2000). The globoid is the mineral storage compartment consisting of mineral-phytate inclusion crystals. Studies from energy-dispersive X-ray analysis in rice and synchrotron soft X-ray microscopy in wheat suggested that minerals and phosphorus are co-localized with the globoid structures in the AG (Ogawa *et al.*, 1979; Regvar *et al.*, 2011). However, during the sample preparation for transmission electron microscopy, the mineral-phytate crystals in the globoid can be easily dissolved out, thus leaving an electron-transparent internal globoid cavity in most of the electron microscopic analyses (Jacobsen *et al.*, 1971). Despite these differences, some of the LAG in *ta2-1* accumulate protein that is stained by CBB, suggesting they are still functioning as storage compartments.

PAS and CBB staining also suggested that the distribution patterns of AG change from outer to inner layers of the *ta2-1* aleurone, with AG resembling wild-type structure in the outer layers, but LAG with empty or limited protein matrix predominant in the inner layers. The increased size of *ta2-1* aleurone cells and subcellular aleurone grains may signal a potential decrease in protein and mineral storage capacity from the outer to inner layers *ta2-1* aleurone.

Hypothesis 2: The aleurone development does not synchronize with the nutrient accumulation during grain development.

From 3-5DAA is the endosperm cellularization stage, during which cells at the periphery first take on distinguishable aleurone features. From 6-9DAA, the aleurone and starchy endosperm cells continue to divide and expand during the endosperm differentiation stage (Olsen *et al.*, 2008; Wu *et al.*, 2016). The proportion of aleurone tissue to starchy endosperm tissue is high in these early stages as compared with the mid and late grain developmental stages with limited cell division. This may explain why thick-aleurone was observed in both wild type and *ta2-1* up to 10DAA. Our histological analyses suggested that aleurone development in *ta2-1* occurs simultaneously with aleurone in the wild type throughout all phases of grain development, so it is likely biosynthetic activity and nutrient accumulation will be synchronized with the normal activities during grain development.

Of these hypotheses, the first appears to have relevance regarding nutrients synthesized in the aleurone, and requires further investigation.

Future Perspectives

The embryo is high in vitamin B1, vitamin E, and lipid, (Juliano, 1993), so that any changes in embryo composition would also affect the overall wholegrain nutritional profile. Future studies should separate and study the composition of aleurone-, starchy endosperm-, and embryo- enriched fractions of *ta2-1*. It may also be possible to use synchrotron analysis to explore compositional differences between the outer and inner layers of the ta2-1 thick aleurone, especially in mineral accumulation.

Future studies should also focus on assessing the bioavailabilities of minerals in *ta2-1*. Various digestion models such as *in vitro Caco*-2 cell, *in vivo* animal feeding trial, and human intervention studies should be applied to measure their absorption efficiencies along the gastrointestinal tract.

List Of Abbreviations

AG – aleurone grain

CBB – Coomassie brilliant blue

DAA – days after anthesis

DW – dry weight

FCR – Folin-Ciocalteu reagent

FFA – free fatty acid

HSD – honestly significant difference

LAG – larger aleurone grains

NCD – noncommunicable disease

NNSP – neutral non-starch polysaccharide

ORAC – oxygen radical absorbance capacity

PAS – periodic acid-Schiff

PC – phosphatidylcholine

ta2-1 – *thick-aleurone 2-1*

TAG – triacylglycerol

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The data generated or analysed during this study are included in 1) Supplementary information file in this article and 2) FigShare repository [https://figshare.com/s/0ae0f17be3bf75f2af4e] before publication. Once the manuscript has been accepted, public repository of [10.6084/m9.figshare.12818867] will be available.

Competing interests

RY, XW, CAH, CML, and PJL are co-inventors on a patent for thick aleurone rice for which CSIRO and the Institute of Botany, Chinese Academy of Science are the co-owners.

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Authors' contributions

Conceptualization, CAH, ARB, CML, and PJL; methodology, CAH, ARB, CML, and PJL; microscopic analysis, XW; nutritional analysis, RY; statistical analysis and data visualization, RY; writing—original draft preparation, RY, XW, ARB, and PJL; writing—review and editing, XW, CAH, ARB, CML, and PJL; supervision, CAH, ARB, CML, and PJL; project administration, CML and PJL; funding acquisition, CAH, ARB, CML, and PJL. All authors agree to this statement. All authors read and approved the final manuscript.

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Supplementary information

All the raw data and original images of all the figures and table included in this manuscript are available Supplementary information files in this manuscript.

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