3D visualization and volume based quantification of rice chalkiness in vivo by using high resolution micro-CT

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Abstract

Background: Rice quality research attracts attention worldwide. Rice chalkiness is one of the key indexes determining rice kernel quality. The traditional rice chalkiness measurement methods are mainly based on naked-eye observation or two-dimensional (2D) image analysis and the results could not represent the three-dimensional (3D) characteristics of chalkiness in the rice kernel. These methods are neither *in vivo* thus are unable to provide technical support for high throughput screening of rice chalkiness phenotype.

Results: Here, we introduced a novel method for 3D visualization and accurate volume-based quantification of rice chalkiness *in vivo* by using X-ray microcomputed tomography (micro-CT). This approach not only develops a novel method to measure the rice chalkiness index, but also provides a high throughput solution for rice chalkiness phenotype analysis.

Conclusions: Our method could be a new powerful tool for rice chalkiness measurement, which would greatly help the research of rice chalkiness traits as well as the quality evaluation in rice production practice.

Background

Rice is the staple food for more than two thirds of the population in China and over half of the population in the world, thus previous research on rice grain yield and quality has attracted attention worldwide (Zeng et al., 2017). Rice chalkiness is a major constraint in rice production because it is one of the key indexes determining both the grain quality (appearance, processing, milling, storing, eating, and cooking quality) and the sales price (Fitzgerald et al., 2009; Siebenmorgen et al., 2013). Rice chalkiness is the opaque part of the endosperm in the rice kernel and it is observed to be in white when comparing to the relatively transparent rest part. According to its location in the kernel, chalkiness traits can be grouped into 3 types of white-belly, white-core and white-base (Yoshioka et al., 2007; Bowles et al., 2012). Previous research showed that rice chalkiness formation was related to abnormal carbohydrate metabolism such as cell wall and starch biosynthesis and the opaque part is the location with loosely packed storage starch granules (Xi et al., 2014). The chalky appearance is associated with the development of numerous tiny air spaces between loosely packed starch granules and the resulting change in light reflection (Tashiro and Wardlaw, 1989). Rice chalkiness is a complicatedly quantitative trait which mainly accumulates at the grain filling stage. Therefore, it is controlled by multiple classes of genes involved in assimilate accumulation in endosperm and is also influenced by multiple environmental factors (Lanning et al., 2011; Bowles, 2012; Wada et al., 2019). Although many QTLs controlling rice chalkiness have been reported by various research groups (Li et al., 2014; Qiu et al., 2017; Wang et al., 2018), the underlying molecular mechanism regulating rice chalkiness is far from clear. One of the bottlenecks is the deficiency of accurate and high throughput rice chalkiness quantification methods to support the highly efficient *in vivo* screening for mutants in the rice chalkiness phenotype.

Quantitative indexes describing rice chalkiness mainly include chalky rice rate and chalkiness degree. In general, the operational processes in traditional chalkiness measurement methods are usually normalized by some international standards (e.g. ISO 7301:2011) or local standards (e.g. GB/T 1354–2018) which have played important roles both in commodity inspection and basic researches. Up to date, methods based on naked-eye observation and artificial regionalization are still widely used in rice chalkiness quantification. However, the traditional methods show poor consistency and objectivity. Such time-consuming and non-objective methods could hardly meet the urgent needs for the efficient and accurate measurement of rice chalkiness and the identification of chalkiness related phenotypes. The broken kernels in processes of glum removing and milling would also decrease the measurement accuracy of chalkiness. Moreover, small volume of chalkiness in the center of rice kernel could be hardly observable by naked-eyes, which would also result in reduced chalky rice rate.

To overcome the above limits, some imaging methods based on digital image scanner and image processing software have been developed to measure and categorize rice chalkiness. Rice chalkiness has been scanned into 2D images based on grayscale value differences between chalky and normal regions in the rice kernel, and the chalky part in the kernel has been finely classified and marked in the image (Yoshioka et al., 2007; ISO 7301:2011). Some image processing software have been previously employed to analyze rice chalkiness by using multiple images captured from different angles of milled rice (Yoshioka et al., 2007; Chen et al., 2013; Sun et al., 2014). Scanning electron microscopy has been usually employed to reveal the density of starch at the μm -scaled level and then indirectly reflect the differences between the chalky and normal regions in the image (Li et al., 2014; Yu et al., 2017). Because of the advances in objectivity and accuracy, these image processing methods have been frequently used as alternative methods to naked-eye observation in rice research and breeding programs.

As a matter of fact, rice chalkiness appears as an amorphous cubic structure in the rice kernel, thus measuring the volume instead of the projection area in rice chalkiness quantification is far more meaningful for both the research and practice. Regretfully, through the current image processing methods, rice chalkiness is all measured in 2D. These images of milled rice are captured by digital camera or scanner from outside. Thus the chalkiness on rice surface is easily detected, but the internal chalkiness related characteristics, are hardly revealed. Scanning electron microscopy which has been employed to analyze the compactness of starch accumulation, could indirectly reflect the rough property of rice chalkiness. However, only a very small area of a rice section can be observed in scanning electron microscopy. Neither the chalky boundary could be well distinguished nor the chalkiness volume be quantified. To be able to accurately analyze internal information about location, shape and volume of rice chalkiness, 3D measurement method for rice chalkiness is urgently needed.

In addition, previously reported chalkiness quantification methods are not *in vivo* for rice grains. Rice chalkiness is located in endosperm. To well reveal the chalkiness, processes of glume removing and milling are essential for methods based on naked-eye observation or image scanning. However, these processes are usually accompanied with the embryo destruction, thus the biological activity of the milled rice as a seed would be completely lost. Similarly, scanning electron microscopy method is also

destructive, because the process of nanogold coating in sample preparation and high energy electron impact in scanning seriously reduce biological activity of rice embryo. Therefore, in vivo quantification method for rice chalkiness is also necessary in the basic research fields.

X-ray microcomputed tomography (micro-CT) is a nondestructive imaging technique that can be used to generate a series of consecutively cross-section digital images of a physical object with micrometer- and submicrometer-scale resolution (Starosolski et al., 2015). The absorption of X-rays as an index of an object's physical properties offers the possibility for spatial segmentation based on the matrix X-ray density in biological samples. Through the 3D image reconstructed from these 2D cross-section images, it allows to visualize and quantify X-ray density related biological traits both in 3D and in vivo. Because of its nondestructive characteristics, micro-CT has been previously used to analyze the features of live animal organs/tissues (Liu et al., 2012; Starosolski et al., 2015). Recently, micro-CT has been preliminarily introduced to visualize and quantify morphology characters of plant organs/tissues, such as xylem, root, leaf, flower and grain (Kaminuma et al., 2010; Dhondt et al., 2010; Brodersen et al., 2011; Knipfer et al., 2015; Cuneo et al., 2016; Staedler et al., 2018). It has been also used to study the root architecture and interaction with soil microorganisms (Verboven et al., 2015; Mairhofer et al., 2015; Earles et al., 2018). Several researches have paid attention to the analysis of water and starch distribution in stem of woody plants (Mairhofer et al., 2015). Hence, the once difficult 3D measurement of rice chalkiness and its spatial localization could be explored in vivo through the 3D micro-CT technology.

Here, we employed micro-CT scanning and 3D reconstruction techniques to analyze rice chalkiness. The volume, 3D shape, and location of chalkiness part in the rice kernel were accurately defined. This approach also provided a high throughput solution for rice chalkiness phenotype analysis in vivo and would greatly help the research of rice chalkiness traits.

Methods

Sample collection and preparation

Zhenshan 97B, Xiangzaoxian, X226, X220 and X191 were used to perform X-ray scanning in micro-CT. All seeds were collected from the Rice Germplasm Resource Bank of Hunan Province. Newly mature rice seeds were manually threshed to separate grains. Brown rice was prepared by removing the husk using a Mini De-husker (Taizhou Cereal Instrument Co. Ltd., Zhejiang, China), and the milled rice was prepared by a mini milling machine (LTJM160, Taizhou Cereal Instrument Co. Ltd., Zhejiang, China).

micro-CT analysis and 3D reconstruction of chalkiness

Rice grains were embedded in Super Light Clay (ordered from Alibaba, China). Samples were loaded into SkyScan 1172 Micro-CT (Bruker, Belgium) containing a cone beam X-ray source with < 5- μ m focal spot, and a sealed, fully distortion corrected, air-cooled, 10 Mp, 12-bit CCD camera that is fiber-optically coupled to a Gd₂O₂S scintillator. The samples were positioned at proper distances from the X-ray source according to the sample size and scanning resolution. The X-ray power settings were showed in Tab. 1.

The results were exported to DCM format files, which were imported into the Mimics Innovation Suite Research 19.0 software (Materialise, Belgium). The rice grains and chalkiness were segregated through “segment tools”, and the 3D images were reconstructed through “Calculate 3D tool”.

Table 1
X-ray power settings

Resolution	15 μm	13.5 μm	10 μm	5 μm	2.5 μm
Voltage (kV)	50	50	50	50	50
Electric current (μA)	150	150	120	140	140

Software and computer hardware

Mimics Innovation Suite Research 19.0 and Chalkiness 2.0 were running in a computer powered by 64 bit Windows 10 (Microsoft, USA). To favorably perform 3D reconstruction of rice grains, optimal hardware configuration included an Intel Core i7-9750H CPU (64 bit), 32 gigabytes of memory and a GeForce RTX 2060 graphics card (NVIDIA, USA).

Rice size analysis through Chalkiness 2.0

Rice image (maximum longitudinal section) was imported to Chalkiness 2.0 software (Chen et al., 2011). The grain profiles were auto-recognized and encircled in a red line, and the chalkiness area profiles were auto-regionalized in a white line. The grain size could be auto-calculated through “Calculate” tool and the test report would be generated in an Excel format file.

Seed germination and growth condition

The rice seeds were soaked in Petri dish (9 cm diameter) with two sheets of filter paper and 20 mL sterile water. Petri dish was placed in a constant temperature incubator for 24 hours at 30 °C. Water was removed and the seeds were washed 3 times with sterile water. Then Petri dish was placed in a constant temperature incubator for 12 hours in dark at 30 °C. Seed germination was observed afterwards. In addition, the germinated seeds were planted in soil with 1 L of Kimura B solution and cultured in green house in 16 h light/ 8 h dark at 30 °C. The growth status was observed after 7 days.

Results And Discussion

Quantification processes of rice chalkiness

The traditional methods for rice chalkiness evaluation includes the processes of glume removing and milling, followed by naked-eye observing to count the chalky kernel percentage and judge the chalky rice rate (Fig. 1a). The chalkiness degree is represented by the ratio in percentage for the respective projected areas of chalky part to the whole milled rice kernel (Fig. 1a).

In our proposed method for 3D visualization and quantification of rice chalkiness, the rice grains can be directly used to perform computed tomography in the micro-CT system. Then after, a series of 2D images of cross-section layers were captured (Fig. 1b and c). 3D images of the intact grain, the chalkiness and non-chalkiness parts in the kernel can be respectively reconstructed through a series of procedures by using the built-in software named Mimics Innovation Suite Research (Fig. 1b, Supplemental file 1).

Comparing to the traditional methods, 3D visualization based on micro-CT can easily and accurately define the volume, 3D shape, and location of chalkiness part in the rice kernel. Following the processes of X-ray scanning and 3D reconstruction, we obtained a series of 3D images of rice gain, brown rice and chalkiness part in the rice kernel (Fig. 1b). The volumes of chalkiness part and brown rice were simultaneously calculated by Mimics Innovation Suite Research software and then chalkiness degree was precisely quantified.

3D chalkiness analysis of different chalkiness types

In this study, we collected samples with 4 types of rice chalkiness, i.e. white-belly, white-core, white-whole and white-back, from different cultivated varieties for the volume-based 3D chalkiness quantification (Fig. 2a). Milled rice was scanned by X-ray under 5 μm resolution. The chalkiness areas and their borders were easily observed in cross-sections (Fig. 2b-e). The chalkiness areas in cross-sections were selected and reconstructed through the Mimics Innovation Suite Research software (Fig. 2b-e). The spatial location and shape of cubic chalkiness part were visualized through the reconstructed 3D chalkiness image, and the chalkiness degree was calculated based on the volume data provided by the built-in software (Tab. 2). Furthermore, we compared the images under different scanning resolutions ranging from 2.5 μm to 15 μm . The rice chalkiness could be well revealed under 2.5 μm and 5 μm resolutions (Fig. 2f-g). Under 10 μm and 15 μm resolutions, the cross-section images were a little vague and the reconstructed rice grain image showed relatively low accuracy, but the chalkiness areas still could be roughly distinguished (Fig. 2h-i).

Generally speaking, the higher the resolution was employed, the longer the scanning time was needed. One test under 2.5 μm resolution took more than 30 minutes and generated larger data (more than 30 gigabytes per grain), which is not convenient for subsequent computer processing. Scanning in the resolution range from 5 μm to 10 μm showed relatively high resolution with less time consumption (about 15 minutes) and proper data size (about 10 gigabytes per test). Therefore, resolutions ranging from 5 μm to 10 μm are suggested for high resolution analysis of rice chalkiness, while resolutions ranging from 10 μm to 15 μm are suitable for rough quantification and high throughput analysis of rice chalkiness due to the significantly decreased scanning time.

Table 2
3D chalkiness quantification of rice kernel samples with different chalkiness types

	Volume of grain (mm ³)	Volume of chalkiness (mm ³)	Chalkiness degree (%)
white-belly	9.96	0.54	5.4
white-core	7.91	0.42	5.3
white-whole	13.65	13.65	100
white-back	14.06	0.79	5.6

High throughput analysis of rice chalkiness

According to the international standards (e.g. ISO 7301:2011) or local standards in China (e.g. GB/T 1354-2018), samples for a single test generally require more than 30 rice grains, thus high-throughput analysis of chalkiness is very important. We tested the maximum detectable number of rice grains under different resolutions in one X-ray scan of the micro-CT system. Under resolution lower than 5 μm , multiple rice grains can be completely scanned by X-ray (Tab. 3). Resolutions ranging from 10 μm to 15 μm can be applied in high-throughput analysis of rice chalkiness since the chalkiness can be well visualized through micro-CT (Tab. 3, Fig. 2h-i). In order to conveniently distinguish the grain area borders, we employed Super Light Clay as the supporting substance because its density is far below the rice kernel density and can be easily segmented from cross-sections by using the Mimics Innovation Suite Research software. Through micro-CT and reconstruction, the cubic rice chalkiness parts in as many as 60 rice grains can be well located, visualized and their volume data can be also accurately calculated at the same time. The chalky rice rate can be also calculated through a series of cross-section images (Fig. 3).

Table 3
Resolution of micro-CT and the detectable number of rice grains

Resolution	13.5 μm	10 μm	5 μm	2.5 μm
Detectable number	40–60	20–30	5–10	1
Sample holder	Custom	Custom	Glass tube	Glass tube

in vivo analysis of rice chalkiness

To confirm the potential effects of X-ray on the rice seeds activity, we monitored the seed germination rate and the seedling growth after the micro-CT test procedures. The results indicated that all rice seeds scanned by X-ray in micro-CT could normally germinate, showing no germination and growth defects (Fig. 4a-e). Therefore, micro-CT can be used for *in vivo* analysis of the rice chalkiness.

In addition, to overcome the limitation of micro-CT related software in auto-calculation of grain length and width, we introduced an image processing software Chalkiness 2.0, which is previously developed by

the authors for 2D chalkiness analysis. Length/width ratio represents the axial maximum length to the radial maximum width. When using Super Light Clay to embed multiple rice grains, it is hard to guarantee that the central axis of all rice grains are in the same plane. To obtain the maximum grain length and maximum grain width, thus we reconstructed several cross-sections near the maximum section (Fig. 4f) and then the 3D image was used for rice size analysis (Fig. 4g). Rice kernel length, width and length/width ratio are exported in a pop-up window and the data can be exported to an Excel format file (Fig. 4h).

Conclusion

The traditional rice chalkiness measurement methods are mainly based on naked-eye observation or two-dimensional (2D) image analysis thus could not reflect the cubic characteristics of chalkiness in the rice kernel. Micro-CT was a powerful tool to visualize the inner structure of bio-samples in vivo with micrometer-scale resolution by using X-ray scan and reconstruction techniques. Through 3D reconstruction and volume calculation, we accurately obtained the information about the volume, shape and localization of kernel chalkiness. Moreover, micro-CT is allowed to scan multiple rice grains at once, and the cubic-shaped chalkiness can be visually located in the 3D rice grains. This volume-based quantification of rice chalkiness in vivo is a new method to accurately localize and measure the cubic-shaped chalkiness part in the rice grain. Meanwhile, the simultaneous scanning of multiple grains showed the advantage for high throughput analysis. Our protocol also showed great potential to be applied in chalkiness phenotype screening, quality inspection and non-destructive analysis for other X-ray density related traits. In short, this in vivo 3D visualization and volume-based quantification of rice chalkiness based on high resolution micro-CT, which is a significant improvement to the traditional naked-eye observation methods and 2D imaging methods, would greatly facilitate the chalkiness phenotype screening for basic research programs focusing on the rice chalkiness traits.

Declarations

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Author contributions

Y.S. and L.T.X. designed the experiments, analyzed the data and wrote the manuscript. Y.S. performed the experiments. All authors approved the article.

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Availability of Data and Materials

All data supporting the conclusions of this article are provided within the article and its supplementary files.

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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Figures

Figure 1

a) Traditional quantification processes of rice chalkiness. Chalkiness visualization processes includes glume removing and milling, following by naked-eye observing and then the chalky rice rate is calculated. The chalkiness degree is evaluated by the projected area rate between chalkiness area and milled rice area. b) Processes of micro-CT for rice grain. c) Bruker Skyscan 1172 Micro-CT system.

Figure 2

The shape and location of rice chalkiness. a) milled rice with chalkiness of white-belly (X220), white-core (X226), white-whole (Xiangzaoxian) and white-back (X191) respectively; Cross-section images, reconstructed 3D rice images and reconstructed 3D chalkiness images of milled rice with chalkiness of white-belly b), white-core c), white-whole d) and white-back e) respectively; A cross-section image and reconstructed 3D milled rice kernel with white-belly chalkiness under X-ray scanning with resolution of 2.5

μm f), 5 μm g), 10 μm h) and 15 μm i). Dark areas indicated by arrow red arrow represented the location of rice chalkiness.

Figure 3

High-throughput scanning by X-ray under 13.5 μm resolution. a) Milled rice (Zhenshan 97B); b) Milled rice were embedded in Super Light Clay (about 2 cm $\times$ 2 cm $\times$ 2 cm); c) One cross-section of milled rice grains under 13.5 μm resolution, and the red arrow points represent the location of rice chalkiness; d) Reconstructed 3D image of milled rice embedded in Super Light Clay; e) Reconstructed 3D image of chalkiness. Dark areas indicated by arrow red arrow represented the location of rice chalkiness.

Figure 4

In vivo analysis of rice (Zhenshan 97B) chalkiness. a) 50 matured rice; b) Reconstructed 3D image of rice grain; c) One of cross-sections of rice grain; d) Germination analysis of rice grain after X-ray scanning. e) Growth analysis of rice seedlings after X-ray scanning. Dark areas indicated by arrow red arrow represented the location of rice chalkiness. f) Fifty cross-sections near the maximum section were

reconstructed; g) Scanned image of reconstructed image (Zhenshan 97B); h) Rice size report through Chalkiness 2.0. The values represented the relative length and width.

Supplementary Files

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- [3Dchalkiness.mp4](#)