# VISUALIZATION OF STARCH GRAINS: A RAPID OBSERVATION METHOD TO ISOLATE MUTANTS WITH DEFECTS IN STARCH GRAIN MORPHOLOGY

*R. Matsushima, M. Maekawa and W. Sakamoto*
*Institute of Plant Science and Resources, Okayama University, Kurashiki, Japan*
*E-mail: rmatsu@rib.okayama-u.ac.jp*

## Abstract

Starch is the most important carbohydrate for the human energy source contained in staple cereals and tubers and is also used for food additives such as thickeners and stabilizer. Starch consists of a large number of glucose units joined by glycosidic bonds and synthesized to form starch grains (SGs) inside plant cells. Despite the simple glucose polymer composition of starch, SGs exhibit various morphologies depending on plant species. The morphological variation of SGs supports the wide range of applications of starch, however the underlying molecular mechanisms have not yet been determined. We developed an effective method for preparing thin sections of seed endosperms without chemical fixation and conventional resin embedding that clearly visualize subcellular starch grains. We applied this method to genetic screening to isolate rice mutants in which starch grains were morphologically altered. In five mutants named *ssg* (*substandard starch grain*), increased numbers of small starch grains (*ssg1* to *ssg3*), enlarged starch grains (*ssg4*), and abnormal interior structures of starch grains (*ssg5*) were observed. The observation method is also applicable to other cereal grains, such as barley, wheat and maize. This method will also serve as a useful technique to study the molecular diversity of starch grain morphologies and to monitor the cereal grain qualities.

**Key words**: Endosperm, rice, starch, thin section, grain qualities

## 1.0    Introduction

Starch is a biologically and commercially important polymer of glucose and is synthesized to form starch grains (SGs) inside plastids (amyloplasts) of higher plants. Despite the simple composition of glucose polymers, SGs exhibit various morphologies depending on the plant species (Harz, 1880; Tateoka, 1962). Although SG morphological diversity was reported more than 100 years ago, the underlying molecular mechanisms that account for the differences in morphology have not yet been determined. In this study, we describe a rapid method to prepare thin sections of cereal endosperms without chemical fixation or resin embedding. Using this method, we performed a genetic screen and isolated five rice mutants defective in SG morphologies. Compared to conventional observation methods, the methods presented here are more effective for obtaining clear images of subcellular SGs and are highly suitable for the examination of a large number of samples.

## 2.0    Materials and Methods

Mutagenization was carried out by soaking *lax1* seeds in 1.5% (v/v) methanesulfonic acid ethyl ester (Sigma, Tokyo, Japan). The M2 line derived from a single M1 plant were grown and M2 seeds were collected from individual M1 plants after self-fertilization. Screening was carried out with at least five seeds from each M2 line. Endosperm thin sections from the M2 seeds were prepared by the rapid method. Starch staining was done by immersion in a drop of deionized water containing 40-times diluted Lugol solution (Iodine/Potassium iodine solution). The samples were subsequently examined under a microscope. To obtain thin sections of Technovit 7100 resin of endosperm, approximately 1-mm cubic blocks were cut out from the endosperm of dry seeds and fixed in FAA solution containing 5% (v/v) formalin, 5% (v/v) acetic acid and 50% (v/v) ethanol for at least 12 h at room temperature. Samples were subsequently dehydrated through a graded ethanol series (30% [v/v], 50%, 70%, 90% and 100%) and then embedded in Technovit 7100 resin (Kulzer and Company, Wehrheim, Germany). The embedded samples were cut with ultramicrotome and glass knives and dried on coverslips. Thin sections (approximately 1 ㎛ thickness) were stained with the diluted Lugol solution and subsequently examined under a microscope.

## 3.0    Results and Discussion

We developed a rapid method to prepare thin sections of mature endosperms (Figure 1).   A mature rice seed was fitted into a truncated 200-μL pipette tip (Figure 1A). For rice and barley, truncated 200-μL tips were appropriate. For maize, a truncated 1-mL pipette tip was suitable. The seed-embedded tip was fixed on a block trimmer that was originally developed for resin block trimming for ultramicrotomy (Figure 1B). The fixed seeds on the block trimmer were manipulated under a stereo microscope. The block trimmer was held by third and fourth fingers of non-dominant hand (Figure 1C), while index finger and thumb of dominant hand held a razor blade attached to the seed. During trimming, the blade was kept horizontal to the seed. The blade was also supported by index finger of non-dominant hand to adjust trimming pressure. Trimming generated a smooth surface on the top of the seed, which was exposed approximately 1 mm out of the pipette tip. Thin sectioning was performed as trimming with the same hand positions, but we kept the angle of the blade approximately 30°. The seeds were easily trimmed using a razor blade (Figure 1D), and thin sections were shaved off the endosperms (Figure 1E). Forceps were used to take and place thin sections onto glass slides for staining.

To test the effectiveness of this method, we tried to prepare thin sections of three cereal species: rice, maize, and barley (Figure 2A). Thin sections prepared using the rapid method were stained with iodine and examined under a microscope. The morphologies of SGs were clearly observed. Rice SGs showed compound grains in which smaller granules were assembled. Occasionally, simple grains were also observed. Maize had simple grains that were round and uniform in size. In barley, we observed small (approximately 5 μm in diameter) and larger (approximately 20 μm in diameter) simple grains coexisting in the same cell.

To isolate morphologically distinct SG mutants, we  screened ethylmethane sulfonate (EMS)-treated M2 seeds using the rapid method. We examined at least   five seeds from each M2 line. Of the 1152 M2 lines, five independent mutants with abnormal SG morphologies were isolated. All mutant grains showed chalky endosperms. To obtain fine images of SGs, we chemically fixed endosperms and embedded them in Technovit resin. Technovit sections (approximately 1 μm thick) were prepared and stained with iodine (Figure 2B). Normal SGs were uniform in size and approximately 10-20 μm in diameter. In *ssg1, ssg2,* and *ssg3* mutants, many smaller, simple grains (less than 10 μm in diameter) were observed, in addition to the normal compound grains. In *ssg4* mutants, larger SGs (greater than 30 μm in diameter) were detected. SGs without internal compound structures were observed in *ssg5* mutants. All of these phenotypes were inherited in subsequent

generations.

When *ssg1* was crossed with Kasalath, 20 out of 109 F2 seeds showed the coexistence of simple and compound grains in their endosperms, indicating that *ssg1* segregated as a single recessive allele ($\chi^2$ = 2.6, P = 0.11). *ssg1* was mapped to the middle of chromosome 2 where the *Amylopectin Branching enzyme IIb* gene (*BEIIb*, Os02g0528200) is located (Harrington et al., 1997). We determined the genomic sequence of the *BEIIb* gene from the 5′ UTR through to the 3′ UTR in the *ssg1*, *ssg2* and *ssg3* mutants, and found several base changes. In *ssg1*, two base changes were identified in introns. One was located in the 12th intron splicing acceptor site. The guanine residue (+5789) essential for correct mRNA splicing was replaced by adenine. The *ssg2* mutant also had two base changes. One was located in the 18th exon, which caused an amino acid substitution from proline to leucine. In *ssg3*, a base change was found in the 17th exon, resulting in a glycine to arginine substitution.

## 4.0     Discussion and Conclusions

Many *Poaceae* species have been examined, mostly in extracted conditions, for SG morphologies. We are now reevaluating SG morphologies of the *Poaceae* species using the rapid and resin-embedding methods to verify previous observations and discover novel types of SGs with unique morphologies. Molecular analysis focusing on the mechanisms that determine SG morphology and descriptive analysis of cross-species SG diversity will lead to a more complete understanding of the molecular diversity of SG morphologies. This method will also serve as a useful technique to understand the molecular diversity of starch grain morphologies and to check the cereal grain qualities.

**References**

Harrington, S. E., Bligh, H. F. J., Park, W. D., Jones, C. A. and McCouch, S. R. (1997). Linkage mapping of starch branching enzyme II in rice (*Oryza sativa* L.) and prediction of location of orthologous genes in other grasses. *Theor Appl Genet,* **94**, pp 564-568.

Harz, C. O. (1880). Beiträge zur systematik der Gramineen. *Linnaea,* **43**, pp 1-30.

Tateoka, T. (1962). Starch grains of endosperm in grass systematics. *Bot Mag Tokyo,* **75**, pp 377-383.
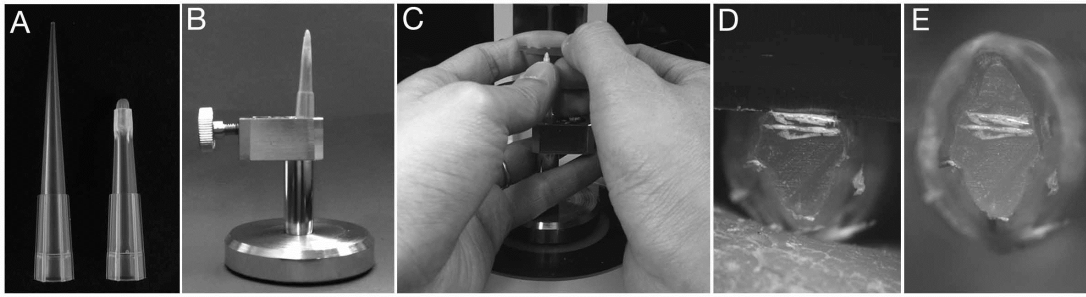
*Figure 1: The rapid method to prepare thin sections of rice endosperms*

A

Rice    Maize    Barley



B

Wild type    *ssg1*    *ssg2*    *ssg3*

*ssg4*    *ssg5*



*Figu*