Imaging Amyloplasts in the Developing Endosperm of Barley and Rice

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Amyloplasts are plant-specific organelles responsible for starch biosynthesis and storage. Inside amyloplasts, starch forms insoluble particles, referred to as starch grains (SGs). SG morphology differs between species and SG morphology is particularly diverse in the endosperm of Poaceae plants, such as rice (*Oryza sativa*) and barley (*Hordeum vulgare*), which form compound SGs and simple SGs, respectively. SG morphology has been extensively imaged, but the comparative imaging of amyloplast morphology has been limited. In this study, SG-containing amyloplasts in the developing endosperm were visualized using stable transgenic barley and rice plants expressing amyloplast stroma-targeted green fluorescent protein fused to the transit peptide (TP) of granule-bound starch synthase I (*TP-GFP*). The *TP-GFP* barley and rice plants had elongated amyloplasts containing multiple SGs, with constrictions between the SGs. In barley, some amyloplasts were connected by narrow protrusions extending from their surfaces. Transgenic rice lines producing amyloplast membrane-localized SUBSTANDARD STARCH GRAIN6 (SSG6)-GFP were used to demonstrate that the developing amyloplasts contained multiple compound SGs.

TP-GFP barley can be used to visualize the chloroplasts in leaves and other plastids in pollen and root in addition to the endosperm, therefore it provides as a useful tool to observe diverse plastids.

Amyloplasts are a type of plastid surrounded by a double lipid bilayer of inner and outer envelope membranes. Plants develop amyloplasts in storage organs such as the endosperm and tubers to biosynthesize and store glucose as starch. Starch is produced in the matrix space (stroma) of amyloplasts and forms particles referred to as starch granules (SGs), which exhibit different morphologies depending on the plant species. The morphological diversity is particularly marked in the endosperm of the Poaceae. SG morphologies are classified as either compound or simple. Compound SGs are formed by the assembly of small starch granules. In rice (*Oryza sativa*) endosperm, compound SGs normally develop to 10–20 μm in diameter, and are composed of individual sharp-edged polyhedral granules with a typical diameter of 3–8 μm; thus, the cross-sections of these compound SGs look like turtle shells. In contrast, simple SGs are composed of single starch granules, and are found in barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), and maize (*Zea mays*). Simple SGs are further divided into bimodal and uniform subtypes; the bimodal subtype comprises smaller and larger simple SGs, known as B-type and A-type SGs, respectively, which coexist in the same cells. The uniform subtype of simple SGs consists of similar-sized granules, which may be hexagonal, pentagonal, or round. Barley and wheat form bimodal SGs and maize produces uniform SGs. Phylogenetic studies have revealed that the compound type is ancestral and found in the majority of the Poaceae family, whereas the bimodal type is specific to a restricted cluster of genera within the Poaceae. The mechanisms controlling the formation of compound and simple SGs have not yet been elucidated.

SGs can be stained violet by iodine to easily visualize their morphologies for standard light microscopy; however, iodine cannot be used to visualize the amyloplast itself, and therefore, amyloplast morphology must be investigated using other methods. For example, GFP-based imaging of amyloplasts has been used for both rice and wheat. In both cases, the transit peptide (TP) of the granule-bound starch synthase I (GBSSI) was fused to the N-terminal end of GFP (TP-GFP). The TP causes the fused GFP to be transported to the surface of the amyloplasts and is cleaved off upon import of the fused GFP across the inner envelope membrane. The GFP can then be observed in the stroma of the amyloplasts and not inside of the starch granules. In wheat, confocal laser-scanning
Visualization of barley amyloplast morphology in early developing endosperm. To visualize barley amyloplasts, we generated a stable transgenic barley plants expressing \( \text{GFP} \) fused with the TP of rice GBSSI (Supplementary Fig. 2a). These transgenic plants, referred to as \( \text{TP-GFP} \), express the chimeric gene \( \text{TP-GFP} \) under the control of the maize \( \text{Ubiquitin 1} \) promoter.

To examine the amyloplasts, we first prepared slices from the early developing seeds of \( \text{TP-GFP} \) barley (Fig. 2a–c). A plot of the sizes of each developing seed (Supplementary Fig. 1) showed that these were similar in size to the wild-type Stage 1 and Stage 2 seeds shown in Fig. 1. Next, we examined the central parts of the seed endosperm using confocal laser-scanning microscopy and obtained differential interference contrast (DIC) and GFP images. In the DIC images, the simple SGs were visible as particles (Fig. 2d–f, left panels). GFP was transported into the stroma of the amyloplasts and clearly revealed the amyloplast morphology (Fig. 2d–f, middle panels). In Stage 1 endosperm, the merged DIC and GFP images showed that the GFP signals surrounded the simple SGs in the amyloplasts (Fig. 2d,e; right panels), and the SGs were observed as black spaces in the amyloplasts in the GFP images (Fig. 2e; middle panel). In most cases, an amyloplast contained multiple simple SGs. In Stage 2, the SGs became larger and the amyloplasts were more elongated than those in Stage 1 (Fig. 2f). Following the growth of SGs, the GFP signals surrounded the SGs became thinner (Fig. 2f, middle panel), and GFP between the SGs within the amyloplasts seemed like concentrated (Fig. 2f, arrowheads). Similar images were observed in rice amyloplasts (Supplementary Fig. 4).

Visualization of barley amyloplast morphology in later developing endosperm. We next observed amyloplasts in later stages of endosperm development. Because the small (B-type) SGs appeared between Stage 3 and Stage 4 (Fig. 1g,h), observation between these stages would be suitable to observe the dynamic changes of SG morphologies. We prepared slices from seeds between Stage 3 and Stage 4 (Fig. 3a–c, Supplementary Fig. 1). Using DIC, we observed both A- and B-types of SGs (Fig. 3d,e, left panels). GFP signals surrounded the SGs (Fig. 3d,e, middle panels), and the GFP-labeled stroma was present in the region between the A-type and B-type SGs (Fig. 3d,e, arrowheads). A z-projection of confocal slices showed that amyloplasts containing B-type SGs were connected to each other by cylindrical GFP signals (Fig. 3f, arrows; Supplementary Movie 1). In Supplementary Movie 1, 3D image obtained from the stack of the seven confocal images used in Fig. 3f. Tiling of the 3D image was shown. B-type SGs were also connected to the amyloplasts containing A-type SGs. These
observations indicate that A-type and B-type simple SGs were present in the same amyloplasts between Stage 3 and Stage 4.

**Morphology of SGs in rice endosperm.** We also examined SGs and amyloplasts from rice using the cultivar Nipponbare. In contrast to barley, flower opening in rice is clearly evident, so the DAF times were easy to determine. We collected developing seeds at 3, 4, and 6 DAF and used them to prepare Technovit thin sections (Fig. 4a–c). We also observed endosperm at 2 DAF (data not shown), but found that endosperm development at this point was insufficient for the observation of SGs using iodine staining. At 3 DAF, small compound SGs were clearly observable in the central part of the endosperm (Fig. 4d). From 3 to 6 DAF, the compound SGs enlarged dramatically (Fig. 4d–f).

**Visualization of amyloplast morphology in developing rice endosperm.** We generated transgenic TP-GFP rice using the same vector used to create the transgenic barley (Supplementary Fig. 2a). We prepared slices from 4-DAF seeds (Fig. 5a), as the seeds at 3 DAF were too small to prepare slices by hand and the seeds at 6 DAF were not suitable for preservation of the integrity of the amyloplast membrane. DIC images of the central part of the endosperm revealed the presence of compound SGs (Fig. 5b, left panel). The GFP localized between the starch granules in the compound SGs in a net-like structure (Fig. 5b, middle panel), and the GFP-labeled stroma was enriched in the regions between the compound SGs (Fig. 5b, arrowheads). This indicates that multiple compound SGs were present in a single amyloplast. Amyloplasts containing two compound SGs were confirmed...
SUBSTANDARD STARCH GRAIN6 (SSG6) is an amyloplast-membrane-localized protein in rice19. SSG6 is thought to control the size of compound SGs, as the ssg6 mutant developed enlarged, spherical compound SGs (Fig. 6a,b)19. We previously constructed a plasmid expressing SSG6 fused with GFP19 (Supplementary Fig. 2b).

When we introduced the SSG6-GFP fusion into the ssg6 mutant (SSG6-GFP in ssg6), the SGs were smaller than those of the parental ssg6 line (Fig. 6c,d, left panels; Supplementary Fig. 5). In addition, the size and shape of SGs were similar between SSG6-GFP in ssg6 and SSG6-GFP in Nipponbare rice (Fig. 6e,f, left panels; Supplementary Fig. 5), indicating that SSG6-GFP was functional. GFP imaging indicated that SSG6-GFP localized to the outer limit of the amyloplast membrane (Fig. 6d,f, middle panels). The GFP signal surrounded the multiple compound SGs, indicating that amyloplasts contain more than one compound SG during seed development.

**Figure 2.** Fluorescence images of the endosperm at Stages 1 and 2 in transgenic TP-GFP barley. (a,b) Stage 1 seeds. (c) Stage 2 seed. (d) Differential interference contrast (DIC), GFP, and merged images of the section prepared from (a). (e) DIC, GFP, and merged images of the section prepared from (b). Arrows indicate amyloplasts containing multiple starch grains (SGs). (f) DIC, GFP, and merged images of the section prepared from (c). Arrowheads indicate GFP-enriched regions inside amyloplasts. (g,h) Transmission electron micrographs of amyloplasts in non-transgenic barley endosperm. Asterisks indicate SGs. Scale bars, 1 mm in (a–c); 10 μm in (d–f); 1 μm in (g,h).
Visualization of plastids in other organs in barley and rice. We were able to clearly visualize chloroplasts in the leaves, amyloplasts in the pollen grains, and plastids in the roots of TP-GFP barley (Fig. 7a–d). In non-transgenic barley plants, no GFP signal was detected under the same detection condition and similar chlorophyll images in leaf and rod-like SGs in pollen grains were observed (Supplementary Fig. 6). In the case of the TP-GFP rice, GFP-labeled amyloplasts in pollen grains were also clearly visualized like endosperms (Supplementary Fig. 7), however we could not obtain clear GFP-images in leaves and roots (data not shown).

Discussion
In this study, we visualized amyloplasts containing SGs using stroma-localized GFP in transgenic barley and rice. We showed that GFP fused with transit peptide of rice GBSSI was functional in barley. This is the first example to develop the stable transgenic plants visualizing amyloplasts in simple-SG-developing plants.

In the developing endosperms of barley and rice, we observed multiple SGs inside individual amyloplasts (Figs 2, 3, 5 and 6). A previous study in transgenic rice had shown that amyloplast division progresses simultaneously at multiple sites and that elongated amyloplasts containing multiple compound SGs undergo several constrictions simultaneously\(^4\). Those results resemble the phenomena we observed in barley and rice, and we therefore concluded that the amyloplasts containing multiple SGs are amyloplasts in the process of division.

In barley, we observed B-type small SGs in the peripheral stroma and GFP-labeled protrusions of the A-type amyloplasts (Fig. 3d–f, middle panels). Similar images were obtained from wheat endosperm transiently expressing TP-GFP\(^12,20\). Considering these similarities, it seems likely that the above characteristics are common features of amyloplasts in species biosynthesizing bimodal SGs, such as barley and wheat. In rice, the budding-type amyloplast division in which large amyloplasts divide by protrusion of small amyloplasts from the surface were also observed\(^14\). Our observation of B-type small SGs in the peripheral stroma and the protrusions from the A-type amyloplasts might be comparable to the rice budding-type amyloplast division. The B-type amyloplasts was budding from A-type amyloplasts and the cylindrical structure might be intermediate process of the budding process.
of B-type amyloplasts from the A-type. Even though SG morphologies were different between rice and barley, the mechanism of amyloplast division process may be common.

Previous observation of the transgenic rice expressing TP-GFP focused on the lateral side of the endosperm (i.e., the subaleurone cells)\(^{11,14}\). The amount of the SGs was less in the lateral part compared to the central part of the endosperm in rice seeds\(^{10}\). In the endosperm of 4-DAF seeds, amyloplasts in the lateral side of the endosperm was small and sparse (Supplementary Fig. 8). Therefore, we observed SG at central part in which SGs could be much more developed. Consistent with previous observation\(^{11,14}\), amyloplast stroma-targeted GFP does not penetrate into the starch granules and therefore localizes around them, where it is observable as a net-like structure in the compound SGs (Fig. 5b). The degree of constriction of amyloplasts in this study was minimal compared to the previous observation of rice amyloplasts in which the constriction was remarkable. This may be because the endosperm was observed at an earlier point in the division process in this study, compared to the previous one\(^{16}\).

To visualize the amyloplast membrane, we introduced the SSG6-GFP gene into wild-type and ssg6 mutant rice (Fig. 6). SSG6 has a putative transmembrane domain and homology to aminotransferases\(^{19}\). The ssg6 mutant develops enlarged, spherical compound SGs in the endosperm (Fig. 6b, left panel). In this study, SSG6-GFP localized at the amyloplast membrane in the ssg6 mutant background (Fig. 6d, middle panel). In a previous study, the fusion protein of an ADP-glucose transporter (Brittle1, BT1) and GFP (BT1-GFP) localized to the outer edge of the amyloplasts, as well as between the granules of the compound SGs\(^{16}\). BT1 is an inner envelope membrane protein\(^{21}\) indicating the presence of an inner envelope-containing structure between the granules\(^{16}\). In contrast, SSG6-GFP was only detected at the outer edge of the amyloplasts (Fig. 6d,f, middle panels), and may therefore be localized at the outer rather than the inner envelope membrane.

We previously developed a simple method for observing SGs in rice\(^{10}\). Using this method, we isolated rice mutants (ssg) with defective SG morphologies. The ssg1, ssg2, and ssg3 mutants had higher numbers of smaller SGs (<10 \(\mu\)m in diameter) in addition to the normal-sized SGs (10–20 \(\mu\)m in diameter). Also, ssg4 and ssg6 developed enlarged compound SGs (>30 \(\mu\)m in diameter), while the SGs of ssg5 lacked the characteristic compound

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**Figure 4.** Starch grains in developing endosperm of wild-type rice cv. Nipponbare. (a) Rice seed at 3 DAF. (b) Rice seed at 4 DAF. (c) Rice seed at 6 DAF. (d) Iodine-stained thin section of endosperm at 3 DAF. (e) Iodine-stained thin section of endosperm at 4 DAF. (f) Iodine-stained thin section of endosperm at 6 DAF. Scale bars, 1 mm in (a–c); 20 \(\mu\)m in (d–f).
**Figure 5.** Fluorescence images of the endosperm in transgenic *TP-GFP* rice. (a) Rice seeds at 4 DAF. (b) Differential interference contrast (DIC), GFP, and merged images of the sections prepared from (a). Arrowheads indicate GFP-enriched regions inside amyloplasts. (c,d) Transmission electron micrographs of amyloplasts in the non-transgenic rice endosperm. (e,f) Magnified images of areas indicated by dotted lines in (c,d), respectively. Constrictions of amyloplasts are visible. Scale bars, 1 mm in (a); 10 μm in (b); 1 μm in (c–f).

**Figure 6.** Fluorescence images of the endosperm in transgenic rice expressing *SSG6-GFP*. (a) Seed of the non-transgenic *ssg6* mutant at 4 DAF. (b) Differential interference contrast (DIC), GFP, and merged images of the section prepared from the seed in (a). (c) Seed of *ssg6* plant expressing *SSG6-GFP* at 4 DAF. (d) DIC, GFP, and merged images of the section prepared from seed in (c). (e) Seed of wild-type plant expressing *SSG6-GFP* seeds at 4 DAF. (f) DIC, GFP, and merged images of the section prepared from seed in (e). Scale bars, 1 mm in (a,c,e); 10 μm in (b,d,f).
SG structure\textsuperscript{10,19,22}. We are now in the process of isolating barley mutants with defective SG morphologies using the same methods as were described for rice. Crossing these barley mutants with the transgenic TP-GFP barley line generated in this study will allow us to characterize the structure of the amyloplasts in the mutants, which may help elucidate the way in which amyloplast morphology is related to the SG defects in the mutants.

Chloroplasts in leaves and plastids in roots had been clearly visualized in rice by the expression of GFP fused with transit peptide of Rubisco Small Subunit\textsuperscript{2} under the control of CaMV 35S promoter previously\textsuperscript{23}. In this paper, other organs except for leaves and roots were observed. In the transgenic TP-GFP plants in this study, TP-GFP was under the regulation of the maize Ubiquitin 1 promoter, therefore, we expected the GFP could be visualized throughout the plants. However, we could not obtain GFP signals in leaves and roots in the T1 transgenic rice. This might be because gene silencing of TP-GFP was occurred specifically at leaves and roots. On the other hand, the transgenic barley plants developed in this study showed GFP-labeled chloroplast and other plastids in leaves, pollens, and roots as well as endosperm (Fig. 7) even after T2 generation. Therefore, this transgenic barley could be used for the further analysis of amyloplasts in endosperm, but also for the study of other types of plastids in whole organs.

Methods

Plant material and growth conditions. Rice (\textit{Oryza sativa} cv. Nipponbare) was grown at 28°C (13-h day/11-h night for the first month then 11-h day/13-h night) in a growth cabinet (#LPH-411S; NK Systems, Japan). Barley (\textit{Hordeum vulgare} cv. Golden Promise) was grown at 15°C/13°C (12-h day/12-h night for the first two months then 16-h day/8-h night) in a growth room. Rice SSG6-GFP plants were constructed as described previously\textsuperscript{19}.

Plasmid construction and transformation. To visualize amyloplasts, a vector was generated containing enhanced GFP (\textit{EGFP}) attached to the sequence of a transit peptide targeted to the amyloplasts, under the regulation of the maize Ubiquitin 1 promoter. The plasmid containing EGFP downstream of the maize Ubiquitin 1 promoter, pBUH3-EGFP, was described in a previous study\textsuperscript{24}. First, the nucleotide sequence

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**Figure 7.** Fluorescence images of chloroplasts in leaves, plastids in pollen and root of TP-GFP barley. (a,b) GFP and chlorophyll autofluorescence and merged images of TP-GFP barley leaves. Differential interference contrast (DIC) image was also shown. (c) GFP, DIC and merged images of TP-GFP barley pollen. (d) GFP, DIC and merged images of TP-GFP barley root. Scale bars, 10 μm.
encoding the transit peptide of GRANULE-BOUND STARCH SYNTHASE I (Os06g0133000) was amplified from the genome using the following primers: 5′-GTTACTTCTGCA GGGGATGGTGCTTGCTCACCGG-3′ and 5′-GCTCACCAGGTTGGGGGTGGCGTACACGACGAC-3′ (the vector-derived sequence is underlined). The obtained Os06g0133000 fragment encodes a portion of the protein from the first methionine to the 79th amino acid. The fragment was cloned into the SacI sites of pBU-HWxTpGFP and used for Agrobacterium tumefaciens-mediated transformation in barley and rice, as described previously25,26.

Preparation of Technovit sections from endosperm. To prepare thin sections of endosperm from dry seeds, approximately 1-mm³ blocks were dissected from the central region of the endosperm and fixed in FAA solution (5% [v/v] formalin, 5% [v/v] acetic acid, and 50% [v/v] ethanol) for at least 12 h at room temperature. To prepare thin sections of developing endosperm, approximately 1-mm³ blocks were dissected from the endosperm and fixed in 3% (v/v) glutaraldehyde in 20 mM cacodylate buffer (pH 7.4) for at least 24 h at 4°C. The method of resin embedding using Technovit 7100 resin (Kulzer, Germany) was described previously27. Thin sections (1 μm) were prepared using an ultramicrotome (LEICA EM UC7; Leica Microsystems, Germany) and diamond blades. The sections were stained as described previously27.

Transmission electron microscopy. Developing endosperm was fixed overnight in 4% (w/v) paraformaldehyde, 2% (v/v) glutaraldehyde in 50 mM cacodylate buffer (pH 7.4) at 4°C, then post-fixed with 2% osmium tetroxide for 3 h at 4°C. The methods used to prepare ultra-thin sections (80–90 nm) were described previously22. The samples were observed using a transmission electron microscope (JEM-1400Plus; JEOL, Japan) at an acceleration voltage of 80 kV. Digital images were captured using a CCD camera (EM-14830RUBY2; JEOL).

Detection of GFP signals in endosperms of transgenic plants. Developing seeds without husks were set in elderberry pith (#1-150-0470; KENIS, Japan) and places in a hand microtome (#3-150-0461; KENIS). Slices were dissected from the fixed seeds using straight razors (#BTM-10H1; KAI Group, Japan). The obtained slices were placed on a glass slide, immersed in a drop of 550 mM sorbitol solution, and covered with a coverslip. GFP signals were detected using a laser-scanning confocal microscope (FV1000; Olympus Corporation, Japan).

Quantification of images. The micrograph images were quantified using Fiji image-processing software28. DIC images were used for the quantification of the SG areas. To discard the SGs oriented in a non-sagittal plane, only the SGs with the two largest areas in each micrograph were used for the quantification.

Observation of leaves, pollen grains and roots. To observe chloroplasts in leaves, approximately 1-mm strips were detached from the third leaves of barley seedling, placed on a glass slide, and immersed in a drop of water. After being covered with a coverslip, the leaves were examined with the laser-scanning confocal microscope. To observe amyloplasts in pollen, anthers just before anthesis were disrupted with forceps in water on a glass slide and released pollen grains were examined with the laser-scanning confocal microscope. Roots of elongation zone at 4 days after germination were examined like leaves.

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Author Contributions
R.M. and H.H. designed this study, performed the experiments, wrote the manuscript, and approved the final version.

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