The amyloplast, a form of differentiated plastid, proliferates in sink tissues, where it synthesizes and stores starch granules. Little is known about the molecular mechanism for amyloplast division and development. The rice (Oryza sativa) endosperm provides an excellent model system for studying molecular mechanisms involved in amyloplast division and starch synthesis. We compared amyloplast division processes in the endosperm of wild type and a mutant of ARCS, a member of the dynamin superfamily. Plant growth and fertility of arc5 were not significantly different from the wild type. Unlike binary fission of chloroplast in the leaf, small amyloplasts in the endosperm of wild type divide simultaneously at multiple sites, generating a beads-on-a-string structure. In addition, large amyloplasts divide by budding-type division, giving rise to small amyloplasts attached to their surfaces. ARC5 and FtsZ2-1 fused to fluorescent proteins were targeted to the constriction sites in dividing amyloplasts. Both the loss of function of ARC5 and overexpression of ARC5 fusion proteins in the endosperm did not produce spherical amyloplasts with increased diameter, but produced either fused amyloplasts with thick connections or pleomorphic types, suggesting that proper stoichiometry between ARC5 and other components in the amyloplast division machinery is necessary for the completion of the late stage of amyloplast division. The size distribution of starch granules purified from arc5 was shifted to small and the starch gelatinization peak temperature was significantly higher than for wild-type starch, suggesting that amyloplast division processes have a significant effect on starch synthesis.

**Keywords:** Amyloplast division • ARCS • Endosperm • FtsZ • Rice • Starch granule.

**Abbreviations:** DSC, differential scanning calorimetry; GFP, green fluorescent protein; IEM, inner envelope membrane; IPTG, isopropyl β-D-thiogalactoside; OEM, outer envelope membrane; ORF, open reading frame; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; RFP, red fluorescent protein.

**Introduction**

The amyloplast in the cereal endosperm is a terminally differentiated plastid where the carbon translocated from source tissues is converted to starch granules and stored until germination. The starch in the cereal endosperm provides not only a major source of dietary energy supply for human and farm animals but also a valuable resource for non-food industries. Despite the enormous importance of the amyloplast as the organelle that synthesizes and stores starch, little is known about how the amyloplast proliferates in the cereal endosperm. The objective of the current study is to obtain a better understanding of the molecular mechanisms for amyloplast division in the endosperm of rice (Oryza sativa). Transient expression of green fluorescent protein (GFP) targeted to the amyloplast in wheat endosperm has revealed interconnections between amyloplasts (Langeveld et al. 2000); this approach demonstrated the feasibility of transgenic analyses in combination with the use of fluorescent proteins for studying amyloplast division in the cereal endosperm. Our stable rice transformation analyses demonstrated that GFP, fused with a transit peptide, is efficiently targeted to the amyloplast in the endosperm (Kawagoe et al. 2005a). The ease of generating stable transformants and the availability of genetic resources make the rice endosperm an ideal model system for studying how the amyloplast divides and develops.
Recent studies on a number of chloroplast division mutants in Arabidopsis (Arabidopsis thaliana) indicate that the plastid division process is coordinated by the action of at least two molecular machineries: an internal machinery on the stromal side of the inner envelope membrane (IEM), and an external machinery on the cytosolic side of the outer envelope membrane (OEM) (Maple and Moller 2007a, Yang et al. 2008). The internal machinery includes FtsZ1 and FtsZ2 proteins (Stokes et al. 2000, Stokes and Osteryoung 2003, Yoder et al. 2007, McAndrew et al. 2008), which are related to the bacterial FtsZ protein involved in cytokinesis (Margolin 2005, Miyagishima 2005). The placement of the FtsZ ring at mid-plastid is dynamically regulated by proteins that include MinD, MinE, ARC3, MCD1, and ARC6 (Fujiwara et al. 2004, Lutkenhaus 2007, Maple and Moller 2007b, Maple et al. 2007, Fujiwara et al. 2008, Nakanishi et al. 2009). In Arabidopsis, the status of the FtsZ ring on the stromal side of the IEM is conveyed across the IEM and OEM through the membrane-associated proteins ARC6, PDV1 and PDV2, which together facilitate the recruitment of ARC5 (DRP5B) to the constriction site on the cytosolic side of the OEM (Miyagishima et al. 2006, Glynn et al. 2008). ARC5 is a member of the dynamin superfamily of eukaryotic GTPases, which form spiral-like structures that pinch membranes (Gao et al. 2003, Hong et al. 2003, Miyagishima et al. 2008). Arabidopsis mutants of ARC5 show a chloroplast division defect in which chloroplasts initiate but rarely complete constriction, generating enlarged, dumb-bell-shaped chloroplasts (Gao et al. 2003).

Each amyloplast in the rice endosperm produces up to several dozen granules, each of which is typically 3–8 µm, polyhedral and sharp-edged. Although rice granules are often described as compound granules, they are not fused or aggregated, and are easily separable by conventional purification procedures (e.g. Fujita et al. 2003). The mechanism preventing fusion of granules within the amyloplast remains elusive. Transgenic potato tubers expressing a high level of StFtsZ1 produce fewer but larger spherical amyloplasts (de Pater et al. 2006), indicating that the size of the amyloplast and hence the granule of the simple type can be modulated by altering the amyloplast division process. An important question is whether amyloplast division mechanisms are different between amyloplasts that produce either simple or compound granules.

In this study, we characterized in detail an arc5 mutant of rice, screened from transposon insertion mutant lines (Miyao et al. 2003), and analyzed the roles of ARC5 in amyloplast division in the endosperm. Unlike the binary fission of chloroplasts, small amyloplasts divide at multiple sites, generating a beads-on-a-string structure. On the other hand, large amyloplasts divide by budding-type division, giving rise to small amyloplasts attached to their surfaces. Inhibition of amyloplast division processes did not produce spherical amyloplasts with increased diameter, but instead produced pleomorphic versions. In addition, we revealed that altering amyloplast division processes has a significant effect on starch synthesis.

### Results

**Rice ARC5 is expressed in the seed but is not essential for seed development**

As a first step towards elucidating the amyloplast division mechanisms in rice endosperm, we chose to characterize a mutant line, NG3642, of rice cv. Nipponbare, in which a transposon is inserted in the fifth exon of ARC5 (Fig. 1A). Rice ARC5 (OsARC5) is presumably a single gene and consists of 17 exons and 16 introns on chromosome 12. The longest open reading frame (ORF) in the full-length cDNA (AK072318) encodes a polypeptide of 775 amino acid residues with a calculated molecular weight of 86,893 Da. OsARC5 contains a conserved dynamin GTPase domain at the N-terminal region and is highly similar to AtARC5 over its entire length. The plant height of arc5 was slightly less than the wild-type control, but seed weight, tiller number and fertility were not significantly different from the wild type when grown in the field (Table 1). The starch content...
Amyloplast division in the rice endosperm

Table 1 The plant growth, fertility and transitory starch content in the leaf blade of arc5

<table>
<thead>
<tr>
<th>Plant</th>
<th>Number of grains per ear (%) (n = 5)</th>
<th>Weight of 100 grains (g) (n = 5)</th>
<th>Number of tillers (n = 20)</th>
<th>Plant height (cm) (n = 20)</th>
<th>Starch content (% fresh weight) (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Fertile: 91.0 ± 1.6 (95.0 ± 0.3)</td>
<td>2.76 ± 0.01</td>
<td>16.3 ± 0.7</td>
<td>102.4 ± 0.5</td>
<td>0.83 ± 0.04</td>
</tr>
<tr>
<td>arc5</td>
<td>Infertile: 83.0 ± 6.8 (87.7 ± 1.4)</td>
<td>2.67 ± 0.02</td>
<td>16.1 ± 1.4</td>
<td>98.4 ± 1.1</td>
<td>0.37 ± 0.02</td>
</tr>
</tbody>
</table>

1Transitory starch content in the leaf blade was measured at the end of the light period and at the end of the dark period. The values are the averages of three replications (mean ± SE). Significant differences from the wild type are marked with a single asterisk (P < 0.01, t-test).

in the leaf blade at the end of the light period was only 45% that of the wild type (Table 1), suggesting that starch synthesis during the day is altered in arc5. We noticed that chloroplasts in the leaf sheath of arc5 seedlings were pleomorphic (Supplementary Fig. S1) and thylakoid membranes were continuous at the constriction sites (Supplementary Fig. S2). These results indicate that ARC5 facilitates chloroplast division in the leaf.

Consistent with the prediction that Tos17 insertion in the fifth exon of ARC5 generated a null mutant, ARC5 protein was not detectable in the seed of arc5 at 10 days after flowering (DAF) (Fig. 1B). The protein levels of FtsZ1 and FtsZ2-1 at 10 DAF were not significantly altered in arc5 (Fig. 1B). We then compared the protein level of isomamylase3 (ISA3), a debranching enzyme targeted to the amyloplast, as a control protein that is not a component of the division machinery. As shown in Fig. 1B, ISA3 levels were not different between wild type and arc5, suggesting that carbon metabolism is not markedly altered in the arc5 endosperm, which is consistent with the fact that seed weight of arc5 was not significantly different from that of the wild type (Table 1). In the developing seed, ARC5 was detectable from 5 to 15 DAF (Fig. 1C), by which time amyloplast division activity is expected to decline to a low level (Hoshikawa 1968). The protein levels of FtsZ1 and FtsZ2-1 were highest at 5 DAF and gradually declined as the seed matured (Fig. 1C). These results show that all three proteins known to play roles in chloroplast division are expressed in the developing seed when amyloplast divisions are active.

Amyloplasts in the endosperm divide simultaneously at multiple sites

Starch in the endosperm is detectable as early as 4 DAF (Xu et al. 2008), indicating that the proplastid differentiates into the amyloplast by that time. Amyloplast divisions in the inner endosperm cells were probably most active before or during the milky stage, which normally ends by 6 DAF. However, the endosperm of the milky stage was difficult to preserve for routine microscopic analysis. We thus compared amyloplast divisions in the lateral side of the outer endosperm (i.e. subaleurone cells), where cell division continues until 9 DAF (Hoshikawa 1967). Because starch granules contain semi-crystalline and insoluble amylpectin, stroma-targeted GFP does not penetrate into the granule and instead occupies the stromal space between the granules (Kawagoe et al. 2005a). In the wild-type endosperm, typical dividing amyloplasts were elongated, with multiple constrictions between the granules (Fig. 2A). As shown in Fig. 2B, dividing amyloplasts were observed to have a beads-on-a-string structure. These results suggest that amyloplast division processes progress simultaneously at multiple sites. At a later stage of seed development, small amyloplasts were found on the surface of larger amyloplasts (Fig. 2C), suggesting that the amyloplast can proliferate by budding-type division. Dividing amyloplasts in arc5 were pleomorphic, as shown in Fig. 2D. The ‘string’ of the beads-on-a-string structure was markedly thick in arc5 (Fig. 2E), suggesting that ARC5 plays a role at the late stages of amyloplast division. In contrast, as shown in Fig. 2F, budding-type division was not severely affected in arc5.

To demonstrate the causal relationship between the mutation of ARC5 and the phenotypes described above, we conducted a complementation assay by expressing DsRed-ARC5 in the endosperm of arc5. When the expression level of the transgene was low and comparable to that of ARC5 in the wild type, most amyloplasts were spherical and indistinguishable from those of the wild type (Fig. 3A–C). In contrast, transformants expressing a high level of DsRed-ARC5 produced pleomorphic amyloplasts (Fig. 3A, D). These results are consistent with the notion that the proper stoichiometry between DsRed-ARC5 and other components of the amyloplast division machinery is necessary for completion of the late stage of amyloplast division.

ARCS is targeted to the constriction sites of dividing amyloplasts

Having shown that ARC5 plays a role in amyloplast division in the endosperm, we then wanted to see the localization of
 ARC5 in the dividing amyloplast. We transformed rice with tpGFP under the control of the rice Glb promoter (Kawagoe et al. 2005a and 2005b) and either DsRed-ARCS or the dominant-negative form DsRed-ARCS(K60A), in which a conserved Lys60 is substituted with Ala (e.g. Arimura and Tsutsumi 2002), under the control of the ZmUbq promoter (Nakamura et al. 2007). As shown in Fig. 4A–C, DsRed-ARCS was concentrated at the constriction sites of dividing amyloplasts. Similar results were obtained with transformants expressing a different pair of transgenes, tpCherry and GFP-ARCS, in the endosperm (Supplementary Fig. S3). DsRed-ARCS(K60A) was also targeted to the constriction sites (Fig. 4D–F). In addition, it was detected as patches on the surface of the amyloplast, indicated by arrowheads in Fig. 4H–I, which could be traces of separation. The negative effect of K60A substitution was relatively mild compared with the dominant effect of the overexpression of DsRed-ARCS in inhibiting division processes of amyloplast.

Mature amyloplasts in the outer endosperm of wild type were spherical and contained compound granules (Fig. 5A) that were generally similar in size in each amyloplast, suggesting that the synthesis of granules is synchronized. Purified starch granules were polyhedral, sharp-edged and separate (Fig. 5D). In contrast, many amyloplasts in arc5 were pleomorphic (Fig. 5B). In particular, putative constriction sites were thick, indicating that the division processes had been arrested. A large fraction of granules purified from the mature seeds of arc5 were irregularly shaped, possibly representing fused granules (Fig. 5E, arrows). The size distribution of the purified granules confirmed that small granules were commoner in arc5 than in the wild type (Fig. 5G).

We then compared starch properties between wild type and arc5 by differential scanning calorimetry (DSC). The gelatinization peak temperature of arc5 starch was significantly higher than for wild-type starch (Supplementary Table S1), indicating that amyllopectin synthesized in arc5 is structurally different from that in the wild type. Overexpression of DsRed-ARCS also inhibited the division processes of amyloplast (Fig. 5C) and chloroplast (Supplementary Fig. S4). Elongated amyloplasts with multiple thick connections (Fig. 5C, arrowheads) and small granules in the vicinity of the arrested constriction sites were characteristic phenotypes of the endosperm expressing a high level of ARCS-DsRed. The size distribution of the purified granules was similar to that of arc5 (Fig. 5G). We noticed that inhibition of amyloplast division in the endosperm did not generate spherical amyloplasts with increased diameter, but instead produced fused amyloplasts with thick connections at putative constriction sites (Fig. 5B, C).

FtsZ2-1 localizes at the constriction sites of dividing amyloplast

One model of chloroplast division processes based on studies on Arabidopsis mutants proposes that FtsZ polymers are stabilized at mid-plastid by a membrane-spanning complex containing ARC6 and PDV2, and that ARC5 is recruited from the cytosol to the constriction site through interaction with PDV1 and PDV2 (Maple and Moller 2007a, Yang et al. 2008). We demonstrated that ARC5 is recruited to the constriction sites in dividing amyloplasts (Fig. 4A–C), and that FtsZ2-1 is

Fig. 2 Dividing amyloplasts in the endosperm of wild type and arc5. GFP with the transit peptide of rice GBSSI at the N-terminus (tpGFP) is efficiently targeted to the amyloplast. Confocal micrographs show that imported GFP is excluded from starch granules and present in the stroma surrounding starch granules. (A)–(C) Confocal micrographs of dividing amyloplasts in the subaleurone cells of the wild type. Constriction sites are indicated by arrows (A and B). Note that the sizes of the amyloplasts in the beads-on-a-string structure shown in (B) are heterogeneous. Budding-type division is indicated by arrowheads in (C). (D)–(F) Confocal micrographs of dividing amyloplasts in the subaleurone cells of arc5. Pleomorphic amyloplasts contain small granules (D), and elongated amyloplasts show thick connections (arrows) that appear to be putative constriction sites where the processes are arrested (E). Budding-type division (arrowheads) appears to be normal in arc5 (F). Bars in (A)–(F) = 5 µm.
Fig. 3 Complementation experiments of arc5 with DsRed-ARC5 expressed under the control of ZmUbq promoter together with tpGFP. (A) Immunoblot analyses with antibodies against OsARC5 and red fluorescent protein (RFP). The expression levels of ARC5-DsRed (arrowheads) of apparent molecular weight 110 kDa are low (+) or high (++) in the developing seed of arc5. The fusion protein was not detected in arc5 transformed with tpGFP alone (−). Unidentified protein recognized by the anti-ARC5 antibody is indicated by an asterisk. (B)–(D) Confocal micrographs of amyloplasts of WT (B), arc5 transformants expressing the fusion protein at low (DsRed-ARC+) (C) or high (DsRed-ARC++) (D) level. The amyloplast morphology expressing the fusion protein at low level (DsRed-ARC+) in arc5 seed (C) is indistinguishable from that of the wild type (B). In contrast, amyloplasts are pleomorphic when the fusion protein is overexpressed in arc5 (D). Note that the starch granules in the amyloplast indicated by an arrow in (D) are heterogeneous in size. Bars in (B)–(D) = 5 µm.

Fig. 4 Localization of DsRed-ARC5 and DsRed-ARC5(K60A) in the endosperm. GFP signals in the stroma (A, D, G) and the fluorescence signals of DsRed-ARC5 (B) and DsRed-ARC5(K60A) (E, H) are converted to green and magenta, respectively, and merged (C, F, I). (A)–(C) DsRed-ARC5 is targeted to the constriction sites (arrows). (D)–(F) The dominant-negative form DsRed-ARC5(K60A) (magenta) is targeted to the constriction sites (arrows in E and F). In addition, patches, as indicated by arrowheads in (H and I), are found on the surface of amyloplasts, which are likely sites of separation. Bars in (A)–(I) = 5 µm.
expressed in the developing endosperm (Fig. 1B, C). These results prompted us to investigate whether FtsZ2-1 protein in the amyloplast stroma is also targeted to the constriction sites. We expressed rice FtsZ2-1 fused to the N-terminus of the DsRed-Monomer under the control of the Zm\textit{Ubq} promoter together with tpGFP. As shown in Fig. 6, the FtsZ2-1–DsRed fusion protein was targeted to the multiple constriction sites in dividing amyloplasts.

**Discussion**

The plastid division proteins \textit{ARC5} and FtsZ2-1 participate in amyloplast division

We compared amyloplast division processes in the endosperm of wild type and a null mutant of \textit{ARC5}, and demonstrated that \textit{ARC5} plays a role in the amyloplast
division processes. Although the growth and fertility of \textit{arc5} were not significantly different from those of the wild type when grown in the field, starch accumulation in the leaf blade of \textit{arc5} during the day was significantly lower than in the wild type (Table 1). It is possible that the movement of chloroplasts in the mesophyll cell for adjusting to different light conditions is partially impaired in the leaf blade of \textit{arc5} due to the pleomorphic shape of the chloroplasts (Jeong et al. 2002, Koniger et al. 2008). Interestingly, both loss of function of ARC5 and overexpression of DsRed-ARC5 resulted similarly in generating pleomorphic amyloplasts in the endosperm (Figs. 2–5). These results suggest that a productive complex of the division machinery is formed only when the stoichiometry of ARC5 relative to other factors is in a suitable range. Consistent with this notion, high expression of DsRed-ARC5 in \textit{arc5} generated pleomorphic amyloplasts, whereas low expression of the fusion gene complemented the division defect in \textit{arc5} mutant (Fig. 3). We showed that FtsZ2-1–DsRed fusion protein was recruited to the constriction sites in dividing amyloplasts (Fig. 6A–C). These results indicate that protein components that polymerize inside IEM (FtsZ2-1) and outside OEM (ARC5) play roles in amyloplast division and suggest that membrane proteins such as ARC6, PDV1 and PDV2, which constitute the complex connecting the FtsZ and ARCS polymers, may also play roles in amyloplast division.

No aplatidial cells have ever been found in the endosperm of \textit{arc5} or transgenic plants overexpressing DsRed-ARC5. It is plausible that proplastid division processes are little affected in \textit{arc5}, considering the fact that plant growth, fertility and seed weight of \textit{arc5} were not significantly different from those of the wild type (Table 1). It is important to note that the Arabidopsis \textit{arc1} mutant accelerates proplastid division, even in those mutants that contain a second mutant gene that completely inhibits the initiation or completion of chloroplast division (Marrison et al. 1999), suggesting that proplastid division depends on a different set of factors from that of the chloroplast and amyloplast division processes. ARC5 may be necessary for the late stage of the division processes for large differentiated plastids such as amyloplasts and chloroplasts, but it may not play a crucial role in the division of proplastids.

Amyloplasts divide at multiple sites and by a budding mechanism

We revealed that the division mechanisms differ between amyloplast and chloroplast. It is plausible that starch granules influence the division processes. Chloroplasts in the rice leaf sheath divide by binary fission (Fig. 7A), in which fission of the thylakoid membrane precedes the separation of the envelope membranes (Supplementary Fig. S2). In contrast, as illustrated in Fig. 7B, the amyloplast division processes progress simultaneously at multiple sites. Elongated amyloplasts containing small starch granules and multiple constrictions were prominent in small, growing endosperm cells in the subaleurone cells. Since budding-type amyloplast division was also found in the \textit{arc5} endosperm (Fig. 2F), the budding process does not appear to depend on ARC5. Similar budding-type division has been found in tomato \textit{suffulta} mutants when chlorophyll-containing chloroplasts degenerate and give rise to chromoplasts in the developing tomato fruit (Forth and Pyke 2006). Note that the overexpression of \textit{AtMinE1} or loss of function of \textit{AtMinD1} leads to a heterogeneous population of chloroplasts by formation of single or multiple FtsZ rings (Fujiwara et al. 2008). MinD and MinE function as a negative regulator of FtsZ polymerization and topological specificity factor essential for chloroplast division site placement, respectively (Maple et al. 2002, Fujiwara et al. 2004, Maple and Moller 2007a, Fujiwara et al. 2008, Nakanishi et al. 2009). We are currently studying possible roles of MinD and MinE in division site placement in amyloplast division in the rice endosperm.

Transgenic potato plants overexpressing potato \textit{FtsZ1} in their tubers have fewer but larger amyloplasts, and hence larger simple starch granules (de Pater et al. 2006). It is thought that increased levels of StFtsZ1 result in partial inhibition.
Fig. 7 Diagrams illustrating the division mechanisms of plastids in rice.
(A) Chloroplasts in the leaf divide by binary fission. (B) Elongated amyloplasts containing small granules generate multiple constriction sites, to which FtsZ2-1 and ARC5 are recruited. This beads-on-a-string structure is seen when the endosperm cell is still small and rapidly expanding its cellular volume. At later stages of endosperm development, large amyloplasts divide by protrusion of small amyloplasts from the surface (budding-type division).

rather than increased plastid division in the tuber. However, in rice endosperm, inhibiting amyloplast division produced pleomorphic amyloplasts that were not necessarily larger than normal amyloplasts. Furthermore, the size of starch granules was, on average, smaller when amyloplast division was inhibited (Fig. 5G). The reason for the marked differences in the effects of inhibition of amyloplast division between rice and potato is not clear, but it is possible that the difference is due to the fact that the rice amyloplast produces compound granules, whereas potato synthesizes simple granules. The gelatinization peak temperature of arc5 starch was significantly higher than for wild-type starch (Supplementary Table S1), indicating that amylopectin synthesized in arc5 is structurally different from that in the wild type. Further studies are needed to investigate the possible relationship between the mode of amyloplast division and the synthesis of compound granules.

Materials and Methods

Materials

Seeds of Tos17-inserted line of ARC5 (NG3642) of O. sativa L. Nipponbare were obtained from the National Institute of Agrobiological Sciences (NIAS; Tsukuba, Japan). Both arc5 and the wild type were grown during the summer under natural environmental conditions in an experimental paddy field. Full-length rice cDNA clones of ARC5 (AK072318), FtsZ1 (AK103448), FtsZ2-1 (AK100526) and ISA3 (AK101554) were obtained from NIAS. The fragments of FtsZ1 (M141–S402, 27.5 kDa), FtsZ2-1 (M211–V452, 25.7 kDa), ARC5 (A499–Q775, 32.0 kDa) and ISA3 (S69–P782, 80.5 kDa) were PCR-amplified and cloned in pET23d (Novagen). The respective polypeptides were expressed in Rosetta 2 (DE3) (Novagen) with isopropyl β-d-thiogalactoside (IPTG) induction, purified from inclusion bodies in a Ni column and injected into rats for antibody production. Anti-RFP antibody was purchased from MBL (Nagoya, Japan). The vectors encoding red fluorescent proteins, pDsRed-Monomer-N1 and pmCherry-N1, were obtained from Clontech.

Plasmid construction and rice transformation

The binary vectors for Agrobacterium-mediated rice transformation were constructed from modified plasmids derived from the Entry and Destination vectors of the Gateway system (www.invitrogen.com), as listed in Supplementary Table S2. The coding sequences were amplified by PCR using templates of rice full-length cDNAs and primers listed in Supplementary Table S3. The ZmUbq promoter (Nakamura et al. 2007) was obtained from Dr. Hiroaki Ichikawa (NIAS, Tsukuba, Japan). The Gt1 terminator was cloned by genomic PCR amplification with PCR primers listed in Supplementary Table S3 and genomic DNA isolated from seedlings of rice cv. Nipponbare. Lys60 of ARC5 was substituted with Ala (K60A) with mutagenic primers (Supplementary Table S3). Transformation of calli derived from rice seeds of wild-type controls (Nipponbare and Yukihikari) or arc5 mutant, and regeneration of plants were conducted as described previously (Kawagoe et al. 2005a).

Total protein extraction and immunoblotting

Seed proteins were extracted from a fine flour of mature hulled seeds by vortexing in extraction buffer consisting of 50 mM Tris–HCl (pH 6.8), 8 M urea, 4% (w/v) SDS, 20% (v/v) glycerol and 5% (v/v) β-mercaptoethanol (35 µl/mg) overnight at room temperature. For protein extraction from immature hulled seeds, samples were homogenized in the extraction buffer (10 µl/mg) using a plastic homogenizer. After centrifugation at 12,000×g for 5 min, proteins were separated by SDS–PAGE in pre-cast 10–20% polyacrylamide gel (ATTO, Japan). The gels were either stained with Coomassie Brilliant Blue or blotted onto a polyvinylidene fluoride (PVDF) membrane for immunoblotting with antibodies and ECL (GE Healthcare).

Laser scanning confocal microscope

Seed without husks were embedded in 5% agarose and cross-sectioned through the middle portion of the seed in 100- to 250-µm-thick sections with a PR07 linear slicer (DSK, Japan). The sections were incubated in phosphate-buffered saline (PBS), and the samples were examined using a TCS SP2 AOBS
laser scanning confocal microscope (Leica Microsystems). The images of dividing amyloplasts are projections of six to eight confocal optical sections taken at 0.7- to 1.0-μm intervals.

**Starch preparation and scanning electron microscopy**

Starch granules were purified from the mature seed of arc5, transgenic rice with a high level of DsRed-ARC5 and wild type (Nipponbare). The mature seed was powdered with a hammer and suspended in 1 ml of the extraction buffer consisting of 55 mM Tris–HCl (pH 6.8), 2.3% (w/v) SDS, 10% (v/v) glycerol and 5% (v/v) β-mercaptoethanol (Echt and Schwartz 1981) for 10 min. The suspension was centrifuged at 2,500×g for 5 min, and the supernatant was discarded. The pellet was washed once with the same extraction buffer, three times with distilled water, and twice with methanol. The starch, resuspended in methanol, was then filtered through a layer of 30-μm mesh and dried. The resulting granules were used to perform scanning electron microscope analysis and DSC. The granules were coated with gold–palladium and the mounted specimens were observed under a scanning electron microscope (S-2380N, Hitachi, Ltd., Japan) at an accelerating voltage of 20 kV. The sizes of 500 granules for each sample were measured.

**Differential scanning calorimetry**

Starch thermography was recorded by DSC (DSC6000S; Seiko Instruments Inc., Japan) as described previously (Umemoto et al. 2004) with some modifications. Purified starch (5 mg) was mixed with 45 μl distilled water. The heating rate was 3°C/min over the temperature range 10–120°C.

**Supplementary data**

Supplementary data are available at PCP online.

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**References**


Supplementary data are available at PCP online.


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