

The Endosperm Morphology of Rice and its Wild Relatives as Observed by Scanning Electron Microscopy

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Abstract While cultivated rice, *Oryza sativa*, is arguably the world's most important cereal crop, there is little comparative morphological information available for the grain of rice wild relatives. In this study, the endosperm of 16 rice wild relatives were compared to *O. sativa* subspecies *indica* and *O. sativa* subspecies *japonica* using scanning electron microscopy. Although the aleurone, starch granules, protein bodies and endosperm cell shapes of the cultivated and non-cultivated species were similar, several differences were observed. The starch granules of some wild species had internal channels that have not been reported in cultivated rice. *Oryza longiglumis*, *Microlaena stipoides* and *Potamophila parviflora*, had an aleurone that was only one-cell thick in contrast to the multiple cell

layers observed in the aleurone of the remaining *Oryza* species. The similarity of the endosperm morphology of undomesticated species with cultivated rice suggests that some wild species may have similar functional properties. Obtaining a better understanding of the wild rice species grain ultrastructure will assist in identifying potential opportunities for development of these wild species as new cultivated crops or for their inclusion in plant improvement programmes.

Keywords Scanning electron microscope · Rice · Wild rice · *Oryza sativa* · *Oryza* species · Endosperm · Aleurone

Abbreviations

SEM Scanning electron microscopy

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Introduction

Cultivated rice, *Oryza sativa*, belongs to the tribe Oryzae which consists of 12 genera. In the genus *Oryza*, there are 22 species of which only two, *O. sativa* and *Oryza glaberrima*, are cultivated. The remaining species within the *Oryza* are commonly designated as wild rices (Vaughan 1994). The *Oryza* grow in diverse locations and conditions, from the wettest areas in the world to the driest deserts, expanding from 53° north to 40° south, from sea level to an altitude of 3,000 m above sea level (Childs 2004; Juliano 2004). Due to their long history of growing under diversified climatic and environmental conditions, a wide range of diversity exists in the *Oryza* genus (Childs 2004). This diversity is particularly abundant amongst the wild species which have not undergone human selection (Vaughan 1994).

Table 1 Wild species of rice studied and their collection record

Taxon	Plant collection record	Place of collection
<i>O. alta</i>	AusTRCF 309269	Guyana
<i>O. australiensis</i>	AusTRCF 309314	Australia
<i>O. glumaepatula</i>	AusTRCF 309281	Suriname
<i>O. grandiglumis</i>	AusTRCF 310560	Brazil
<i>O. nivara/O. sativa</i> (<i>O.</i> hybrid)	AusTRCF 309308	Myanmar
<i>O. latifolia</i>	AusTRCF 309288	Guatemala
<i>O. longiglumis</i>	AusTRCF 309291	Indonesia
<i>O. meridionalis</i>	AusTRCF 300119	Australia
<i>O. meyeriana</i>	AusTRCF 309296	Malaysia
<i>O. granulata</i>	AusTRCF 309284	India
<i>O. officinalis</i>	AusTRCF 309302	Myanmar
<i>O. nivara</i>	AusTRCF 309299	Myanmar
<i>O. rufipogon</i>	AusTRCF 309317	Australia
<i>O. spontanea</i>	AusTRCF 309310	Nepal
<i>O. sativa</i> cv. Nipponbare	N/A	Australia
<i>O. sativa</i> cv. TeQing	N/A	Australia
<i>P. parviflora</i>	AusTRCF 318105	Australia
<i>M. stipoides</i>	AC04-1003504	Australia

Several traits such as resistance to abiotic and biotic stresses have been successfully introgressed from wild species into cultivated rice, resulting in improved cultivated varieties (Vaughan 1994). However, wild species may have potential as new crops in their own right with intrinsically similar starches and functionalities to cultivated rice. Analysis of the functional and nutritional properties of the grain of these ‘wild species’ is required before they can be considered for commercial use. A previous study on grain morphology of wild species of *Oryza* (Kasem et al. 2010) reported comparable grain size and shape of wild *Oryza* to those of cultivated rice. Another study (Shapter et al. 2008) reported similar endosperm morphology of wild species of rice to that of *O. sativa*.

The endosperm plays an important role in determining the nutritional value of rice through its component parts; starch, protein, lipids and fibre (Kang et al. 2006). Starch granule morphology and the physicochemical characteristics of starch influence each other, which in turn affect the total functionality of starch and the procedures that are employed in starch refining (Singh and Kaur 2004). While starch granule shape and size are not the only factors that affect starch performance, they are believed to be a contributing factor to starch functional properties (Singh and Kaur 2004).

Many scanning electron microscope (SEM) investigations of rice starch morphology along with the starch physicochemical properties have been undertaken (Jane et al. 1994; Kang et al. 2006; Sodhi and Singh 2003; Watson and Dikeman 1977), a few of which have been comparative studies of selected wild (mainly *Zizania aquatica*, *O. australiensis*, *O. rufipogon*) and cultivated rice starch

granules (Hoover et al. 1996; Lorenz 1981; Patindol et al. 2006; Shapter et al. 2008; Wang et al. 2002). Little comparative morphological information is available on a comprehensive set of rice wild relatives.

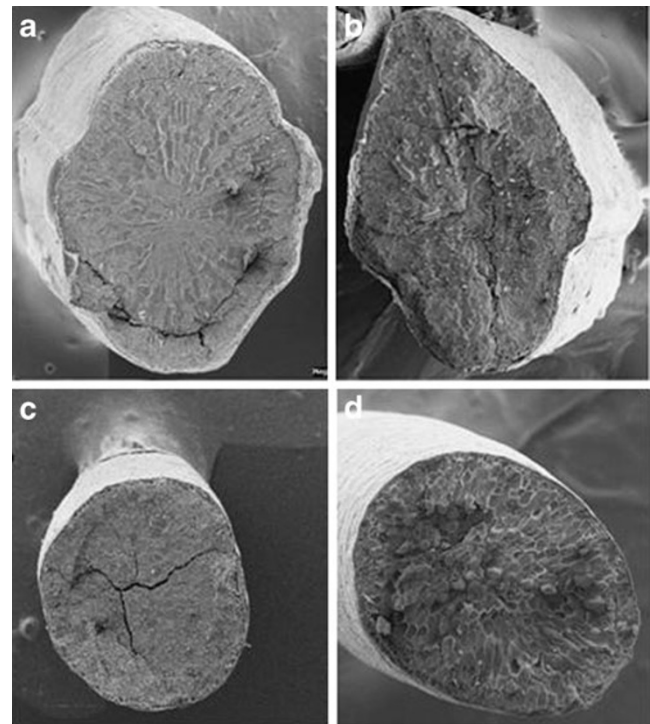


Fig. 1 Representative images of a transverse cross-section of rice and its wild relatives. **a** *O. sativa* cv. Nipponbare; **b** *O. alta*, **c** *O. longiglumis*, **d** *M. stipoides*.

In this study, the aleurone and endosperm of a wide range of wild and cultivated rice grain were compared using scanning electron microscopy. Obtaining a better understanding of wild rice grain ultrastructure will assist in identifying novel wild rice species grain traits and starch properties that could have potential commercial applications.

Materials and methods

Plant material

Grains of wild *Oryza* species and *Potamophila parviflora* were obtained from the Australian Tropical Crops and Forages Collection, Department of Employment, Economic Development and Innovation, Biloela, Australia (<http://www2.dpi.qld.gov.au/>, last accessed on 1 July 2010). *Oryza* hybrid is a cross between *Oryza nivara* and *O. sativa* (interim designation used in GRIN for *Oryza* hybrids which lack an acceptable binomial; <http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?318042>, last accessed on 1 July 2010). *Microlaena stipoides* was sourced from the Australian Plant DNA Bank, Southern Cross University, Lismore,

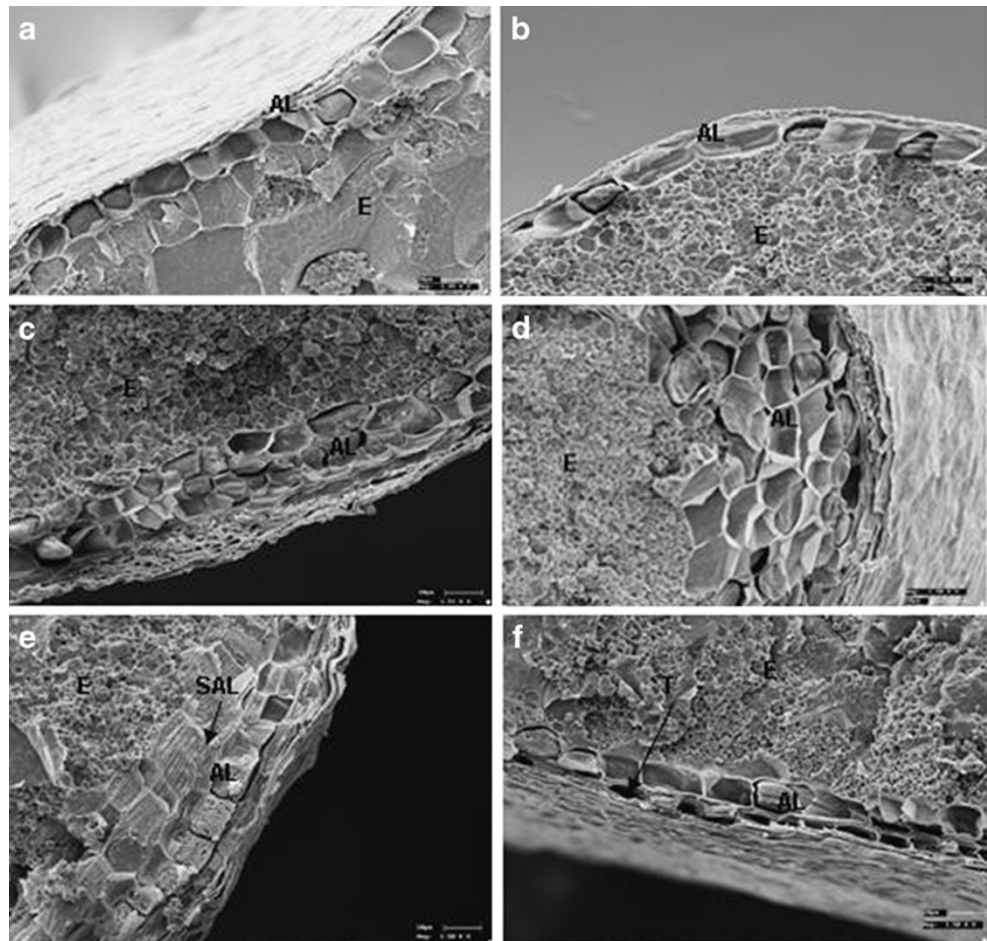
NSW, Australia. Cultivated *Oryza* species *O. sativa* cultivar cv. TeQing and *O. sativa* cv. Nipponbare were sourced from Yanco Agricultural Institute, Yanco, NSW, Australia. The list of wild rice species and their collection record appear in Table 1.

Scanning electron microscope

Grains were manually de-husked and fixed for 24 h in 10% formalin. Fixed grains were rinsed three times for 30 min with distilled water. Rinsed grains were then dehydrated by a standard series of ethanol washes: 25%, 50%, 75% and 95% each for 20 min and then three washes of 30 min each in 100% ethanol. Samples were immediately dried using a Polaron E3100 Critical Point Drier (ProSciTech, Thuringowa, QLD, Australia).

Critical point dried samples, with the exception of *P. parviflora*, were snapped in half transversely using two pairs of forceps. The tiny grain size of *P. parviflora*, precluded this approach so these samples were cut using a scalpel. Halved grains were mounted on aluminium stubs with the fractures facing up. Specimens were gold coated for 30 s at 35 mA. Images were recorded on a Leo440

Fig. 2 Representative images of aleurone layers in rice and its wild relatives. **a** Lateral, one-cell thick cubic cell shaped, *O. latifolia*; **b** ventral, one-cell thick elongated cell shape; *O. latifolia*, **c** dorsal, two- to three-cells thick *O. glumaepatula*, **d** dorsal, five-cells thick, *O. sativa* cv. Nipponbare, **e** subaleurone layer, *O. spontanea*, **f** Testa cell, *O. alta*. AL, aleurone layer; E, endosperm; SAL, subaleurone layer; T, testa.



Stereoscan SEM with the probe current at 20 pA, EHT at 10.00 kV and a working distance of 15 mm.

Results and discussion

Transverse sections

Most of the *Oryza* grain had an undulating perimeter exhibiting up to seven lobes (Figs. 1a and b). In contrast, *Oryza longiglumis* and the two non-*Oryza* species (*P. parviflora* and *M. stipoides*) had simple continuous perimeters without lobes (Figs. 1c and d). *Oryza alta*, *O. nivara*, *Oryza officinalis*, *Oryza spontanea*, *Oryza glumaepatula* and *O. sativa* cv. TeQing had similar perimeters with fewer lobes and a narrower ventral side (Fig. 1b) in comparison with *O. sativa* cv. Nipponbare, *Oryza* hybrid, *Oryza granulata*, *Oryza latifolia*, *Oryza meyeriana*, *Oryza meridionalis* and *Oryza grandiglumis* which had more lobes and a wider ventral side (Fig. 1a).

Aleurone, pericarp and testa

The aleurone surrounds the endosperm, is one to several cells thick depending upon the species, and

can influence milling quality (Stone 1996). In accord with previous reports, aleurone cell shape varied between the species studied, as did the number of cells in this layer. Within the *Oryza*, the number of cells in the aleurone differed from one to five depending on the position either dorsal, ventral or lateral (Fig. 2 and Table 2). The dorsal side usually exhibited more cell layers than the ventral or lateral sides. In *O. sativa*, up to seven dorsal cell layers have been reported (Watson and Dikeman 1977; Zhou et al. 2002; Juliano 1972), however, we found five dorsal cell layers which is in accord with Wada and Lott (1997). *O. meridionalis* had a similar number of cells in the aleurone to *O. sativa* cv. Nipponbare while *O. sativa* cv. TeQing, *Oryza rufipogon*, *Oryza australiensis*, *O. nivara*, *O. meyeriana* and *O. glumaepatula* had three dorsal cell layers suggesting these non-domesticated species may have similar milling properties to cultivated rice. Aleurone cell shapes were not uniform in these species having both elongated and cubic-shaped ventral and lateral cells, while the dorsal cells were always irregular (Table 2).

The aleurone of the one *Oryza* species, *O. longiglumis*, and two non-*Oryza* species, *P. parviflora* and *M. stipoides*, were composed of a single cell layer, with uniform cell shapes throughout.

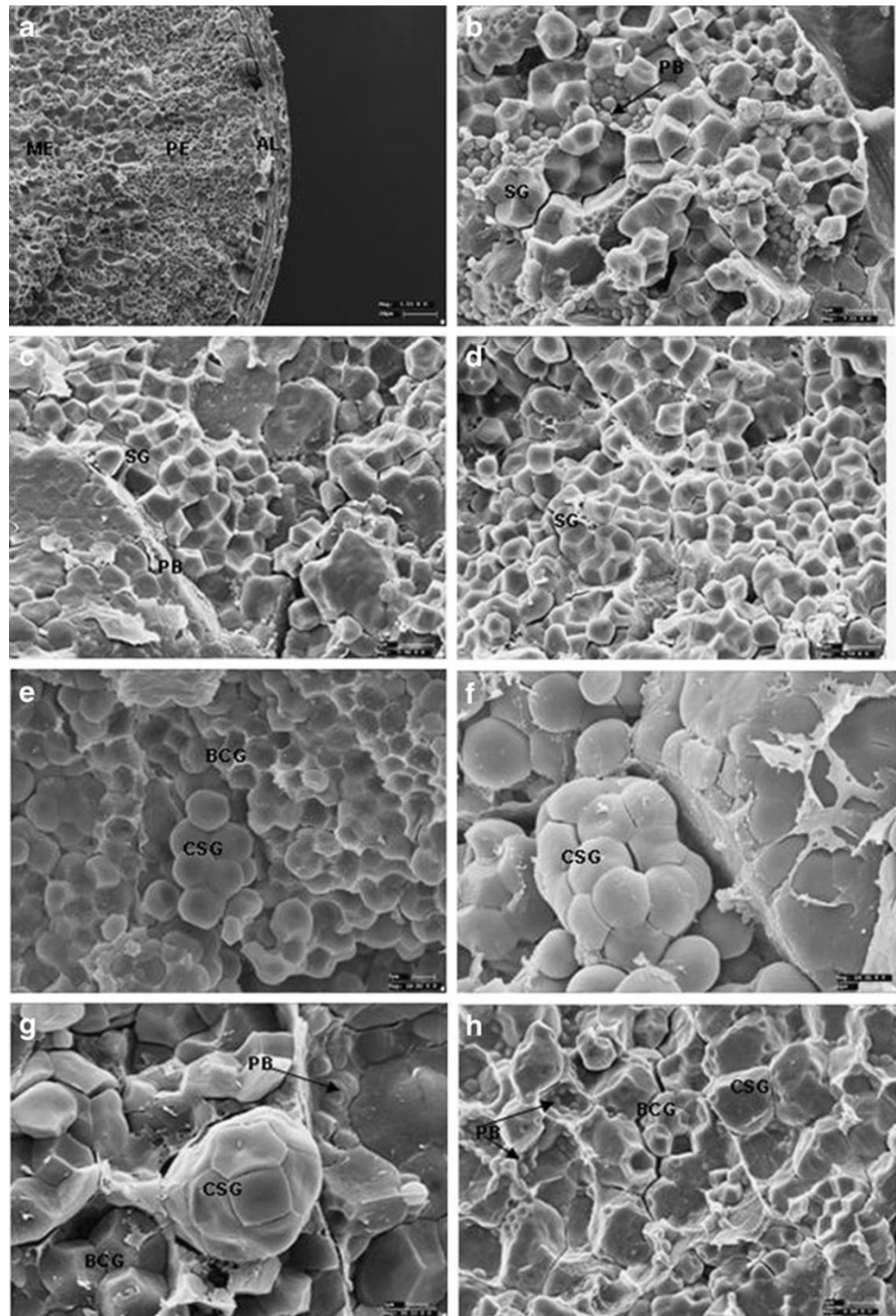
Table 2 Aleurone morphology of rice wild relatives

Species	Dorsal cell thickness	Ventral cell thickness	Lateral cell thickness	Dorsal cell shape	Ventral cell shape	Lateral cell shape	Pericarp thickness
<i>O. sativa</i> cv. Nipponbare	5	1–2	1	Irregular	Cubic and elongated	Mostly elongated	thick
<i>O. sativa</i> cv. TeQing	2–3	1	1	Irregular	Elongated		thick
<i>O. nivara</i> / <i>O. sativa</i> (<i>O.</i> hybrid)	3	1	1	Irregular	Elongated	Cubic and elongated	thicker
<i>O. nivara</i>	2–3	1	1	Irregular	Elongated	Cubic and elongated	
<i>O. granulata</i>	2	1	1	Irregular	Elongated	Elongated	thick
<i>O. latifolia</i>	1–2	1	1	Irregular	Elongated and cubic	Mostly Cubic	thin
<i>O. spontanea</i>	2	1–2	1	Irregular	Elongated	Cubic	
<i>O. alta</i>	1–2	1	1	Mostly elongated some cubic	Elongated	Elongated	thick
<i>O. australiensis</i>	2–3	1	1	Irregular	Cubic, elongated and triangle	Cubic	Thick testa evident
<i>O. officinalis</i>	2	1–2	2	Irregular	Elongated	Elongated	
<i>O. rufipogon</i>	2	1	2	Irregular	Cubic	Cubic	
<i>O. grandiglumis</i>	1–2	1	1	Irregular	Elongated	Mostly elongated	thick
<i>O. meyeriana</i>	2–3	1	2	Irregular	Cubic	Elongated	thick
<i>O. glumaepatula</i>	2–3	1	1	Irregular	Elongated	Elongated	thick
<i>O. meridionalis</i>	3–4	1–2	1	Irregular	Elongated	Cubic	thick
<i>O. longiglumis</i>	1	1	1	Cubic	Cubic	Cubic	thin
<i>P. parviflora</i>	1–2	1–2	1–2	Mostly elongated	Mostly elongated	Mostly elongated	thin
<i>M. stipoides</i>	1–2	1–2	1–2	Cubic	Cubic	Cubic	thin

For most of the species, no clear subaleurone was evident; however, a distinctive subaleurone was observed in *O. spontanea* and *O. latifolia* (Fig. 2e). The wild relatives of sorghum also have a distinctive subaleurone (Shapter et al. 2009) and it is correlated with high protein in rice (Bechtel and Juliano 1980) and other cereals (Shewry and Morell 2001).

The pericarp and testa had a similar thickness among most of the *Oryza* and conformed to the typical Gramineae arrangement, being fused together (Stone 1996). However, a clearly visible testa was observed in *O. alta*, *O. australiensis* and *O. grandiglumis* (Fig. 2f). In *O. longiglumis* and the remaining non-*Oryza* species, the pericarp and testa appeared more compressed, and therefore thinner than the other *Oryza* species.

Fig. 3 Representative images of the endosperm of rice wild relatives. **a** An overview of endosperm from periphery towards central zone, *P. parviflora*, **b** frequent presence of protein bodies at the periphery of endosperm, *O. grandiglumis*, **c** moderate presence of protein bodies at the zone in between central and periphery of endosperm *O. grandiglumis*, **d** almost no protein bodies at the centre of endosperm, *O. grandiglumis*, **e** spherical and smaller compound granule near to the edge of endosperm, *O. granulata*, **f** compound granules towards the centre of endosperm, *O. australiensis*, **g** compressed compound granules transitioning to angular shape, *O. grandiglumis*, **h** compound granules are more intact and angular in the central endosperm, *O. glumaepatula*. *AL*, aleurone layer; *PE*, endosperm near periphery; *ME*, endosperm between centre and periphery; *SG*, starch granule; *PB*, protein bodies; *IN*, indentations; *CSG*, intact compound starch granule; *BCG*, broken compound granule.



Changes across the endosperm

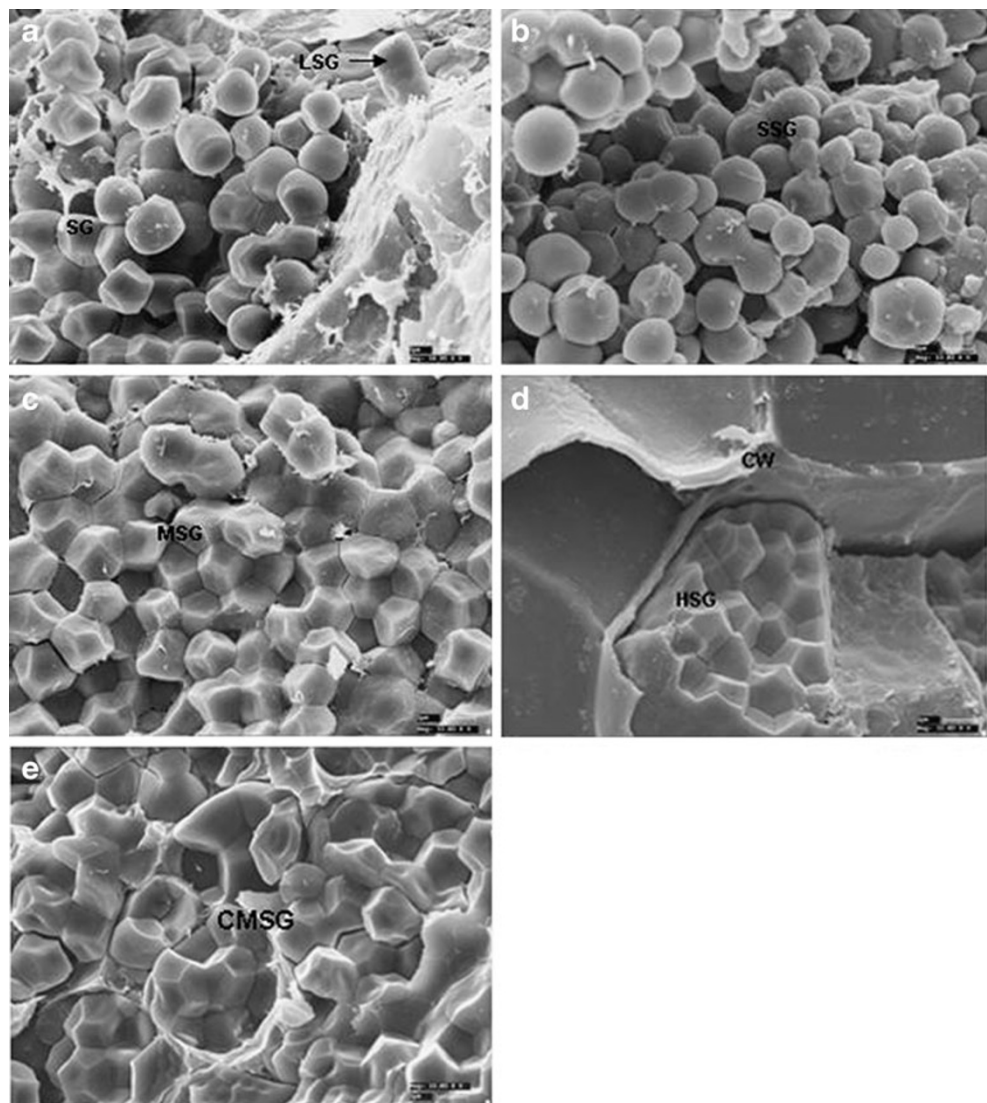
The endosperm cells maintained a radial orientation consistent with previous reports (Evers and Millar 2002) but their shape varied between elongated and non-elongated. *O. granulata*, *O. meyeriana*, *O. latifolia*, *O. officinalis*, *O. glumaepatula*, *O. meridionalis*, *O. sativa* cv. TeQing, *O. sativa* cv. Nipponbare and *O. rufipogon*, had elongated cells while the endosperm of the remainder of the *Oryza* and *M. stipoides* were non-elongated. Dang and Copeland (2004) found the endosperm cells of waxy rice varieties were more elongated than those from the non-waxy varieties and within the waxy varieties, medium grained waxy varieties had longer cells compared to long grained waxy varieties.

Low numbers of intact endosperm cells were observed in *O. latifolia*, *O. meridionalis*, *O. glumaepatula* and *O. grandiglumis*. The spillage of endosperm cell contents

commonly observed in these species could be due to the effect of the fracturing process or a soft endosperm and/or less adhesion between starch granules in amyloplasts. Therefore, the lack of intact cells could indicate that these species have softer grains. *P. parviflora* and *O. longiglumis* cell shape could not be recorded as no intact endosperm cells were found.

In accord with previous findings (Leesawatwong et al. 2004; Pal et al. 1999), protein bodies occurred most frequently at the periphery when compared to the central endosperm (Figs. 3b–d) in all species. A high concentration of protein bodies at the periphery may mean these grains are less susceptible to breakage during milling (Leesawatwong et al. 2004). Protein bodies of the wild species were of a similar size to that of cultivated rice, generally varying from 0.5 to 2.5 μm . Most species had both large and small protein bodies embedded in a thin protein matrix. Indentations on compound starch granules due to the presence of protein

Fig. 4 Representative images of starch granules in endosperm of rice wild relatives. **a** Lenticular-shaped starch granule in *O. latifolia*, **b** spherical-shaped starch granule *O. meyeriana*, **c** mildly angular starch granule *O. alta*, **d** highly angular starch granule *M. stipoides*, **e** compressed starch granule *P. parviflora*. SG, starch granule; LSG, lenticular starch granule; SSG, spherical starch granule; MSG, mildly angular starch granules; HSG, highly angular starch granules; CMSG, compressed starch granules; CW, cell wall.



bodies were also observed (Fig. 3h). Protein bodies were visible between starch granules and were spherical or ovoid in shape. Some individual protein bodies were joined to each other by very fine fibrils forming protein body clusters which were then joined by a protein matrix or cell membrane (Fig. 3a).

Visually *O. australiensis*, *O. meyeriana* and *O. longiglumis* exhibited the fewest protein bodies among the *Oryza* species. Even at the periphery of the endosperm of these species, the presence of protein bodies were almost unnoticeable. Among the cultivated varieties, TeQing exhibited the greatest abundance of protein bodies. Among the wild species, *O. officinalis* had the highest occurrence of protein bodies with a high concentration in the central zone of the endosperm, suggesting this could be a high protein species. *P. parviflora* exhibited a low frequency of protein bodies throughout the endosperm while none were observed in *M. stipoides*.

Starch granule size and shape

The diversity of amyloplast sizes and shapes was such that no clear patterns were evident which would allow the placement of the species into discrete categories. A gradation in the size of compound granules (Fig. 3e–h) was detected from periphery to the centre of the endosperm. The starch granules of both cultivated and wild rice fell in a similar size range of 2–7 μm . Generally, smaller loosely packed compound granules were found at

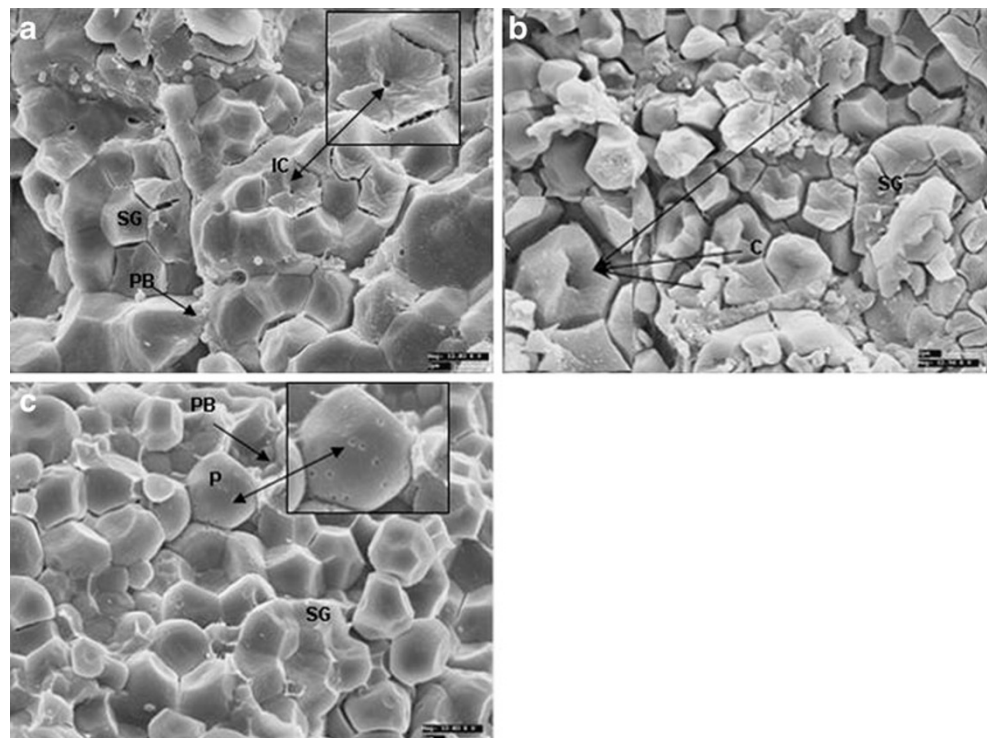
the periphery of the endosperm while larger, compressed compound granules were towards the centre of the endosperm in all species (Figs. 3e–h). Different sized starch granules were often observed in the same compound granules (Fig. 3h).

In general, starch granules were polygonal with edges that ranged from smooth to highly angular and arranged in compound granules (Figs. 4a–e). Spherical starch granules were usually found at the periphery of the endosperm and they gradually increased in angularity towards the centre. Individual oval or lenticular starch granules were occasionally observed in *O. australiensis*, *O. latifolia* and *O. sativa* cv. TeQing. Both spherical and angular polygonal starch granules were present in *O. spontanea*, *O. granulata* and *O. hybrid* (*O. nivara/O. sativa*).

The starch granules of *M. stipoides* were polygonal with sharply defined edges (Fig. 4d). Reichert (1913) reasoned that polygonal granules with sharply defined edges were caused by the pressure of numerous starch granules present in the endosperm cells. Earp et al. (2004) suggested tight packing of starch granules in *Sorghum bicolor* resulted in more sharply edged granules compared with granules that are loosely attached to each other.

O. meyeriana (Fig. 4b) was unique in that it showed spherical starch granules throughout the endosperm. In the compound granules, the individual starch granules were found loosely attached to its neighbouring granules and most of the compound granules were also loosely attached to its neighbouring compound granules. This loose attach-

Fig. 5 Representative images of channels, pinholes and internal cavities in the starch granules of rice wild relatives. **a** Internal cavities in starch granules, *O. australiensis*, **b** channels in starch granules, *P. parviflora*, **c** pin holes on starch granules, *O. rufipogon*. SG, starch granule; C, channels; P, pinholes; IC, internal cavities; PB, protein bodies.



ment may explain why they retained their spherical shape. Some soft wheat varieties have loosely packed starch granules in their endosperm and lower gelatinization temperature than those of hard varieties (Gaines et al. 2000; Zeng et al. 1997).

P. parviflora (Fig. 4e) had starch granules which were unlike those of any other species. The starch granules were compressed with depressions on their lateral sides which may have resulted from pressure from surrounding starch granules.

Starch granule pin holes and channels

Pinholes and channels have been reported in sorghum, barley, oat, corn and millet (Earp and Rooney 1982; Fannon et al. 1992, 2004; Glaring et al. 2006; Shapter et al., 2008) but not rice. Reichert (1913) made a passing reference to ‘cavities’ in *Microlaena* and *Oryza* species but did not clearly describe their appearance. Channels were found in the starch granules of *P. parviflora* and occasionally in *O. grandiglumis* (Fig. 5b). Other *Oryza* species had internal cavities which were only noticeable when the fracture went through rather than around the starch granule (Fig. 5a). Pin holes across the starch granule surface were observed in *O. rufipogon*, *O. spontanea*, *O. latifolia* and *O. meyeriana* (Fig. 5c).

Pin holes, channels and internal cavities facilitate enzyme susceptibility and chemical reactivity (Fannon et al. 1992) and because of this have a positive impact upon the production of modified starches (Fannon et al. 1992, 1993, 2004). Species with pores, channels and cavities are therefore potential sources of starch which will be amenable to enzyme manipulation for a range of industrial uses. It could be anticipated that the porosity of the starch granules might aid the digestion of these grains in the human or animal gut.

Observations of the grain ultrastructure of the rice wild relatives suggest that these species have potential as human food and/or as sources of starch for industrial use. The aleurone, starch granules and protein bodies were similar to cultivated rice while the pores, channels and internal cavities observed in some species may be desirable for both food uses and modified starch production. Further characterisation of the functional qualities and end use characteristics of these wild species could provide incentives for the development of new rice cultivars in the future.

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