Seed Science Research (2006) **16**, 223–227 DOI: 10.1079/SSR2006249

SHORT COMMUNICATION

Mechanical and enzymatic separation of ripening rice (*Oryza sativa* L.) caryopsis tissues

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Abstract

The ripening rice (*Oryza sativa* L.) caryopsis contains several maternal and embryonic tissues that transport assimilates along the partitioning pathway. Experimental access to transport steps in the pathway is limited by the separability of the tissues. Hence, the extent to which tissues can be mechanically and enzymatically separated was assessed. The caryopsis coat was isolated during mid-ripening, dissected and microscopically characterized. In centripetal order, the pericarp (epidermis, parenchyma-cell layer, cross-cell layer, tube-cell layer), inner integument, nucellar epidermis, aleurone layer and subaleurone layer adhered to each other. There was mechanical separation at the tube-cell layer, but not at the maternal/embryonic interface. Aleurone- and subaleurone-layer cells exposed on the inner surface of isolated caryopsis coats were macerated with Pectolyase Y-23 and manually sheared, which freed the endosperm and exposed the nucellar epidermis. Yield of endosperm cells increased linearly with the number of coats and reached a maximum after 1 h. The proportion of cells that were viable was approximately 10%. Subaleurone-layer cells contributed less than 25% to the yield. These results suggest that pectin polymers are necessary for adherence between the nucellar epidermis and the aleurone layer, and that enzymatic maceration is useful for separating the tissues at the maternal/embryonic interface.

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Keywords: adhesion, caryopsis coat, cell isolation, grain ripening, maceration, *Oryza sativa*, rice

Introduction

The rice (*Oryza sativa* L.) caryopsis, a seed surrounded by a pericarp, is composed of layers of embryonic and maternal tissues (Bechtel and Pomeranz, 1977; Hoshikawa, 1993; Krishnan *et al.*, 2001; Ishimaru *et al.*, 2003; Krishnan and Dayanandan, 2003). A dorsal vascular bundle runs longitudinally through the pericarp. In regions of the caryopsis lateral to the dorsal vascular bundle, the starchy endosperm, subaleurone layer, aleurone layer, nucellar epidermis, inner integument, tube-cell layer, cross-cell layer, parenchyma-cell layer and epidermis adjoin in centrifugal order (Hoshikawa, 1993). The maternal nucellar epidermis and embryonic aleurone layer compose the maternal/embryonic interface.

Rice grain ripening involves transport of partitioned assimilates through, and accumulation of dry matter in, the caryopsis. Assimilates, imported via the phloem, move circumferentially through the nucellar epidermis and radially into the endosperm. Plasmodesmata accommodate symplasmic movement of assimilates between nucellar epidermis cells, but not from the nucellar epidermis to the aleurone-layer cells (Oparka and Gates, 1981a, b). Hence, the nucellar epidermis must release nutrients into the apoplasm, before the aleurone layer takes them up.

Experimental systems that result from grain dissection are used for transport studies. Aleurone layers with attached maternal caryopsis tissues (Phillips and Paleg, 1970) and caryopsis segments have been adopted for studies of enzyme secretion from the aleurone layer of mature caryopses. Lacking the embryo, the aleurone layer is the only living tissue in these systems, since maternal tissues die at maturity (Hoshikawa, 1993), and the endosperm cells undergo

programmed cell death (Young and Gallie, 2000). However, ripening caryopses contain several living tissues in addition to the aleurone layer.

Tissue adherence limits the usefulness of dissection for the preparation of more tissue-specific systems. For instance, during development, the outer integument degenerates, and the inner integument fuses to the inner surface of the pericarp, or tube-cell layer (Hoshikawa, 1993). As a result, it is challenging to separate the seed, which takes up nutrients and deposits storage compounds, from the rest of the fruit, which supplies the seed. In addition, the aleurone layer adheres to the maternal tissues of the seed. Furbank *et al.* (2001) have measured sucrose uptake by slices of developing caryopses, but point out that the results lack tissue specificity.

Enzymatic techniques may allow for greater separation of caryopsis tissues. Cells can be isolated enzymatically (Zaitlin, 1959; Zaitlin and Coltrin, 1964; Takebe *et al.*, 1968), pectin-degrading enzymes have been employed on a number of fruits and vegetables for cell separation and isolation (Nakamura *et al.*, 1995); and aleurone-layer cells have been isolated from imbibed grains (Taiz and Jones, 1971; Eastwood, 1977; Hooley, 1982).

Identification of transport mechanisms that function along the transport pathway is essential for analysis of the control of dry matter accumulation. However, experimental access to the transport pathway can be difficult because of the complex caryopsis anatomy (Fisher and Oparka, 1996). Thus, we asked how practical it would be to prepare tissues for the study of transport at the maternal/embryonic interface of the ripening rice caryopsis. Our objective was to assess the extent to which mechanical and enzymatic separation of caryopsis tissues is possible. Tissues were dissected, enzymatically macerated and characterized by light microscopy. Enzymatic, but not mechanical, separation of the tissues at the maternal/embryonic interface was achieved.

Materials and methods

Rice growth

Rice (*Oryza sativa* L., cv. Jefferson) was grown in a greenhouse. Day and night temperatures were set to 30 and 25°C, respectively. Supplementary lighting ($12\,h\,d^{-1}$) was provided by 400-watt high-pressure sodium lamps. Reverse osmosis water was used. Every week, 12 pots (8″ Regal Standard, ITML Horticultural Products, Inc., Brantford, Ontario, Canada) were filled with moistened potting soil (Metro-Mix 360, Sun Gro Horticulture, Vancouver, British Columbia, Canada). The soil was amended with iron chelate (Sprint, Becker Underwood, Inc., Ames, Iowa, USA; $1\,g\,pot^{-1}$), Osmocote (The Scotts Company, Marysville, Ohio, USA; $1\,g\,pot^{-1}$) and water-soluble fertilizer (20–10–20, Peat-Lite Special, J.R. Peters, Inc., Allentown, Pennsylvania, USA; $300\,ml\,pot^{-1}$, 200 ppm N), and pots were arranged in four trays kept full with water. Two grains were planted 1 cm deep at the centre of each pot. After 3 weeks, the pots were thinned to one seedling pot^{-1}, and water-soluble fertilizer was applied weekly, as above.

Microscopic characterization of the caryopsis coat

The isolated caryopsis coat was washed by rubbing its inner surface gently under running cold water. Wet mounts of coats and cross-sections (1 mm thick) embedded in wax were viewed with a normal and a water-immersion objective, respectively, under bright field and fluorescence illumination. Stains were used at the following concentrations: 0.05% (w/v) Janus Green; 0.005% (w/v) Fast Green; 0.5% (w/v) I_2 + 1% (w/v) KI; 0.01% (w/v) fluorescein diacetate (FDA) dissolved in dimethylsulphoxide (DMSO) and diluted 100-fold with water.

Results and discussion

The caryopsis coat isolated during mid-ripening was frontally separable from within the vascular tissue and tube-cell layer into outer and inner coats

Caryopses were isolated from mid-ripening panicles 10–14 d after emergence. One side lateral to the dorsal vascular bundle was cut lengthwise using a razor blade, and the starchy endosperm and embryo were removed using the fingertips. The structure that remained weighed 7.9 ± 0.2 mg ($n = 30$), had a surface area of approximately $30\,mm^2$, and was termed the caryopsis coat.

Further dissection frontally separated the caryopsis coat into two halves, termed the outer and inner coats (Fig. 1). The caryopsis coat halves were separated by applying light pressure to the caryopsis coat and swirling it in a puddle of water on a hard surface. Lateral regions were easier to separate than dorsal regions.

Because the separated inner and outer layers are thinner and more transparent than the intact caryopsis coat, it is possible to inspect their surfaces and underlying layers microscopically without sectioning. Frontal views of inner and outer coat surfaces showed that the dorsal vascular tissue and lateral tube-cell layer partitioned between both coats. Tissues located

https://doi.org/10.1079/SSR2006249 Published online by Cambridge University Press

to the inside and outside of the tube-cell layer were distributed to inner and outer coats, respectively (Fig. 1). Patches of brightly fluorescent inner integument cells in FDA and green cross-cells were visible from the outer surface of inner coats and inner surface of outer coats, respectively. Hence, separation of the caryopsis coat occurs dorsally within the dorsal vascular tissue, and laterally within the tube-cell layer (Fig. 1). The tube-cell layer has many intercellular spaces, which form when the caryopsis increases in girth (Hoshikawa, 1993). This may explain why separation is favoured there, and why ease of separation increases with lateral position.

Endosperm was present on the inner surface of the caryopsis coat isolated during mid-ripening

Frontal microscopic views showed that adhered endosperm remained on the inner surface of the inner coat. Some maternal cells positioned at the edges of the inner coats did not have adhered endosperm. The aleurone layer was continuous, contained 1875 ± 121 cells mm^{-2} ($n = 10$), and was covered in patches by the subaleurone layer (Fig. 1). Both tissues appeared to be heterogeneous in the number of cell layers. Organelles of the subaleurone layer were brightly fluorescent in FDA, giving it a speckled appearance. Conversely, the aleurone layer displayed a homogeneous fluorescence that was very weak.

Cross-sections of the caryopsis coat showed that the subaleurone layer was thinner than the aleurone layer, and dorsal regions of both layers were thicker than lateral ones. Variation in thickness of the nucellar epidermis was less. The subaleurone layer was contiguous only to portions of the aleurone-layer cross-section (typically less than half of it), while the nucellar epidermis covered it completely.

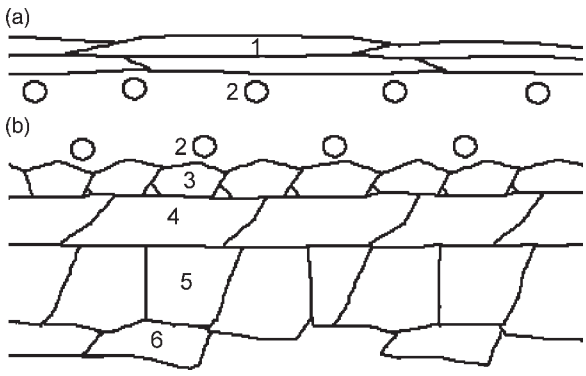

Figure 1. Mechanical separation of rice caryopsis tissues. The caryopsis coat was isolated during mid-ripening and frontally separated. The distribution of tissues lateral to the dorsal vascular bundle is shown in cross-section (not to scale). Outer tissues such as the epidermis are not shown. Separation occurs in the tube-cell layer. (a) Outer coat: 1, cross-cell layer; 2, tube-cell layer. (b) Inner coat: 2, tube-cell layer; 3, inner integument; 4, nucellar epidermis; 5, aleurone layer; 6, subaleurone layer. 1–4, maternal tissues; 5–6, embryonic tissues; 3–6, seed tissues; 1–2, pericarp tissues.

Application of Fast Green and $I_2 + KI$ to caryopsis coats made the subaleurone layer more easily discernible. Its cells were chromophilic to Fast Green and chromogenic (developed black coloration) in $I_2 + KI$. Subaleurone layer cells were more chromophilic and less chromogenic the closer they were to the aleurone layer. The aleurone layer was not chromogenic in $I_2 + KI$ and was chromophobic to Fast Green.

In the context of caryopsis cross-sections, the term 'caryopsis coat' has been defined to include only the maternal tissues (Bechtel and Pomeranz, 1977). Here, in the context of mechanical separation of caryopsis tissues, the term 'caryopsis coat' includes all the tissues that remain after as much embryonic tissue as possible has been removed mechanically from the caryopsis. Use of this definition takes into account the embryonic aleurone and subaleurone layers that adhere tightly to the maternal nucellar epidermis during mid-ripening (Fig. 1). Hence, the mechanically isolated caryopsis coat can contain both embryonic and maternal tissues.

The aleurone layer and nucellar epidermis adhere to each other after the early stages of ripening. This adhesion does not result from cell plate formation during cytokinesis, which is the more common occurrence (Jarvis *et al.*, 2003). Aleurone-layer initials, the peripheral cells that result from the first round of mitosis and cytokinesis during endosperm cellularization (Olsen, 2001), have outer periclinal walls derived from the central cell wall of the embryo sac (Brown *et al.*, 1996). The embryo sac is surrounded by a multi-layered nucellus, which contains inner cells that completely degenerate after aleurone-layer differentiation (Ishimaru *et al.*, 2003). Hence, cells of the aleurone layer abut and tightly adhere to those of the nucellar epidermis. The mechanism of this adhesion at the maternal/embryonic interface is not understood.

Enzymatic maceration separated maternal tissues and adhered endosperm

Isolated caryopsis coats were prepared at a rate of 200 coats h^{-1} and collected in ice-cold incubation medium (IM; 10–20 coats ml^{-1}). IM contained 600 mM mannitol, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mM Mes, adjusted to pH 6 with NaOH (final concentration about 1 mM). Caryopsis coats in IM were transferred to a 50 ml conical centrifuge tube, vortexed for 30 s, transferred to a Buchner funnel and

https://doi.org/10.1079/SSR2006249 Published online by Cambridge University Press

then rinsed with water. The coats were transferred to an equal volume of IM containing 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan), and incubated (room temperature, without shaking) for 1.5 h. Coats were transferred back to an equal volume of IM and manually sheared with a fingertip. The cell suspension was transferred to nylon mesh (105 μm; Small Parts Inc., Miami Lakes, Florida, USA) and the filtrate collected.

Manual shearing of caryopsis coats after 5 min of incubation in Pectolyase Y-23 exposed the nucellar epidermis on their inner surface, except in a few regions still covered by the aleurone layer. Maternal tissues remained largely intact. The nucellar epidermis was chromogenic in Janus Green.

Cells of the adhered endosperm were observed in the suspension obtained following further enzymatic maceration. Only a portion of the cells passed through the nylon mesh. The greatest number of cells was isolated when caryopsis coats were incubated for at least 60 min, and cell yield was linear with the number of coats incubated per volume of IM up to the highest value measured (20 coats ml^{-1}). Approximately 56,000 cells were isolated per caryopsis coat. Some maternal tube and cross cells (Fig. 1) were also observed in the suspension.

Endosperm cells were commonly observed still attached to each other, but the aggregates usually had fewer than five cells. Although the cell aggregates were fewer than single cells, they contained the majority of cells (Fig. 2).

Cells were stained in suspension with Fast Green, I_2 + KI and FDA at concentrations given above in the Materials and methods. Concentrations and dimensions of cells were determined with a Neubauer haematocytometer and an ocular micrometer, respectively. Subaleurone-layer cells were distinguishable from aleurone-layer cells by being chromophilic to Fast Green and chromogenic in I_2 + KI. They contributed less than 25% to the yield of isolated endosperm cells, and had a greater product and ratio of length and width (Table 1). Cytoplasmic granules released by subaleurone-layer cells were commonly observed. About 10% of the isolated endosperm cells fluoresced in FDA (Table 1). The fluorescence of aleurone-layer cells in suspension was more distinct than that of the aleurone layer of caryopsis coats. Some cells within an aggregate fluoresced while others did not. Tube and cross cells did not fluoresce.

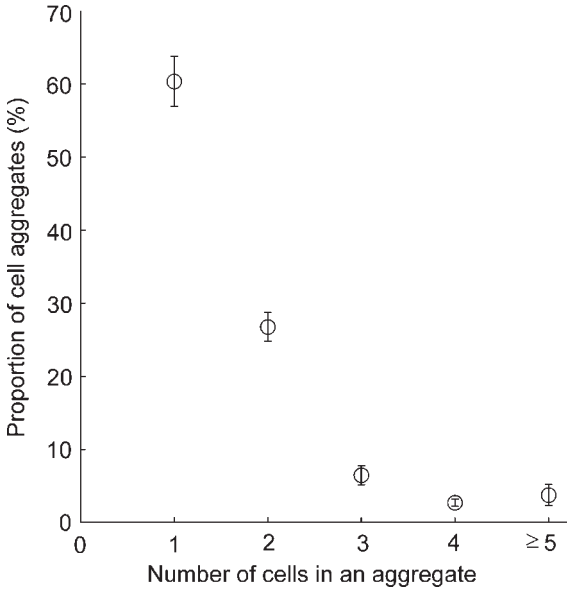

Figure 2. Proportion of isolated endosperm cell aggregates with various sizes. Bars are the SE of a mean of nine replicates.

Endosperm cells were suspended in solutions of KCl (0.2–1 M). A small proportion of the cells plasmolysed appreciably when exposed to KCl solutions greater than 200 mM. These cells appeared shrivelled and were fluorescent when also exposed to FDA. The proportion of cells showing this characteristic increased with higher concentrations of KCl, and reached a maximum of approximately 15% around 800 mM.

Enzymatic maceration enabled complete separation of endosperm and maternal tissues. This method for separation is useful because the nucellar epidermis and aleurone layer of rice are not separated *in vivo* by an endosperm cavity which, in wheat, has been taken advantage of experimentally to assess transport of assimilates (Wang *et al.*, 1993). Furthermore, from our experience, mechanical separation of the maternal/embryonic interface in rice was not possible after the early stages of ripening, making enzymatic isolation a useful alternative.

Table 1. Proportion and size of isolated rice endosperm cells with various staining characteristics (means and SE)

Staining characteristic	Proportion of cells (%), $n = 8$	Cell length × width (μm^2), $n = 20$	Cell length/width, $n = 20$
Fast Green-chromophobic (aleurone layer)	78.7 ± 1.6	604 ± 44	1.3 ± 0.07
Fast Green-chromophilic (subaleurone layer)	21.3 ± 1.6	1005 ± 167	1.6 ± 0.1
I_2 + KI-chromogenic (subaleurone layer)	15.0 ± 1.1	1513 ± 188	1.4 ± 0.06
FDA-positive	9.7 ± 2.3	Not measured	Not measured

https://doi.org/10.1079/SSR2006249 Published online by Cambridge University Press

Conclusion

Adhesion among tissues within the caryopsis coat is a major factor that limits the extent to which they can be separated. Mechanical separation of the maternal/embryonic interface is not feasible during mid-ripening. This complicates tissue-specific assessment of transport steps. The problem of tight adhesion between the aleurone layer and nucellar epidermis was circumvented here by enzymatic maceration and isolation of the cells exposed on the inner surface of isolated caryopsis coats. Two new experimental systems result: isolated aleurone-layer cells and caryopsis coats free of embryonic tissues. The isolated aleurone-layer cells can be used to prepare protoplasts for the study of sucrose uptake (Hay and Spanswick, unpublished).

Acknowledgements

The authors thank the College of Agriculture and Life Sciences for support, and Professors Larry Walker, Susan McCouch and J. Derek Bewley for helpful comments.

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Received 27 September 2005
accepted after revision 29 May 2006