RESEARCH PAPER

The role of the sucrose transporter, OsSUT1, in germination and early seedling growth and development of rice plants

Graham N. Scofield1, Naohiro Aoki1,*, Tatsuro Hirose2, Makoto Takano3, Colin L. D. Jenkins1 and Robert T. Furbank1,†

1 CSIRO Plant Industry, Canberra, ACT 2601, Australia
2 Department of Rice Research, National Agricultural Research Center, Joetsu, Niigata 943-0193, Japan
3 National Institute of Agrobiological Sciences, Tsukuba 305-8602, Japan

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Abstract

Using expression analysis, the role of the sucrose transporter OsSUT1 during germination and early growth of rice seedlings has been examined in detail, over a time-course ranging from 1 d to 7 d post-imbibition. Unlike the wheat orthologue, TaSUT1, which is thought to be directly involved in sugar transfer across the scutellar epithelium, OsSUT1 is not expressed in the scutellar epithelial cell layer of germinating rice and is, therefore, not involved in transport of sugars across the symplastic discontinuity between the endosperm and the embryo. OsSUT1 expression was also absent from the aleurone cells, indicating it is not involved in the transport of sucrose in this cell layer during germination. However, by 3 d post-imbibition, OsSUT1 was present in the companion cells and sieve elements of the scutellar vascular bundle, where it may play a role in phloem loading of sucrose for transport to the developing shoot and roots. This sucrose is most likely sourced from hexoses imported from the endosperm. In addition, sucrose may be remobilized from starch granules which are present at a high density in the scutellar ground tissues surrounding the vasculature and at the base of the shoot. OsSUT1 was also present in the coleoptile and the first and second leaf blades, where it was localized to the phloem along the entire length of these tissues, and was also present within the phloem of the primary roots. OsSUT1 may be involved in retrieval of sugars from the apoplasm in these tissues.

Key words: Germination, OsSUT1, rice, sucrose transport, sugar uptake.

Introduction

During germination and early growth of the rice plant, the developing heterotrophic embryo depends entirely upon carbohydrate reserves remobilized from starch granules stored predominantly in the endosperm. This carbon supply must be transported from the endosperm to the coleoptile and roots, where it is required for metabolism in these actively developing and growing vegetative sink tissues. Early work in rice by Murata et al. (1968) and Palmiano and Juliano (1972) indicated that the starch is broken down, by the action of α- and β-amylases, predominantly into the reducing sugar glucose, and low amounts of other reducing sugars such as maltose and the non-reducing sugar sucrose. Nomura et al. (1969) proposed that the glucose produced in the endosperm moves to the scutellum where it is converted to sucrose, the form in which the carbohydrate is then transported to the growing sink tissues of the shoot and roots.

Further evidence to support the theory that the scutellum is a site for sucrose synthesis was provided by analysis of sucrose-phosphate synthase (SPS), a key enzyme in the synthesis of sucrose. The rice SPS enzyme was first identified and isolated from rice scutellar tissue by Nomura and Akazawa (1973, 1974). It was suggested that sucrose may be synthesized both from starch grains in the scutellum and from glucose transported into the scutellum from the endosperm. More recently,
Chávez-Bárcenas et al. (2000), using in situ RT-PCR and SPS promoter-GUS methods, showed that SPS expression is localized solely to the scutellum of germinating rice grains. They also demonstrated that the activity of SPS significantly increases in germinating rice at 2–3 d after sowing, and appears to be co-ordinated with the activity of α-amylase and is independent of light. In addition to the endosperm, the aleurone layer may also be a source of sucrose for the developing and growing embryo. Chrispeels et al. (1973) reported that, in barley, the aleurone contains high amounts of sucrose and that the aleurone layer was also a site of sucrose synthesis. They demonstrated that sucrose levels in the aleurone decline during seed germination and that sucrose release from the aleurone is regulated by gibberellic acid.

The pathway that the remobilized carbon takes to the sink tissues may include steps that involve either symplastic transport between cells via plasmodesmata connections and/or transport across the apoplasm between cells. In the latter case, sugar transport across membranes may be mediated by energy-dependent sugar transporter membrane proteins. The scutellar epithelial cell layer provides a symplastic discontinuity to sugar uptake, and represents a step in the pathway where sugar transporters may be involved. Once sugars have been taken up across the epithelium they must be transported via the scutellum parenchyma to the scutellar vasculature which connects to the phloem of the developing coleoptile, leaves, and roots. In maize, Humphreys (1987) reported that a symplastic pathway exists for sucrose movement from scutellar parenchyma cells to sieve tubes in the scutellar vasculature. On reaching the scutellar vasculature the sucrose must be loaded into the phloem for transport to the developing shoot and root.

From previous work there is evidence to support the involvement of sucrose transporters (SUTs) during germination and the early stages of plant development. OsSUT1 is expressed in germinating rice seed and within etiolated shoots of young rice plants (Hirose et al., 1997; Aoki et al., 2003). Furthermore, knock-out of OsSUT1 function through anti-sense suppression resulted in a reduction and/or delay in the rate of germination and early growth (Scofield et al., 2002). Expression analysis by RT-PCR indicated that, of the five members of the rice SUT gene family, OsSUT1 is highly expressed and may play a major role in germination, whereas transcript levels of the other four members of the gene family were relatively low compared with their expression in other tissues (Aoki et al., 2003). In germinating wheat seed (Aoki et al., 2006) the presence of the TaSUT1 transcript and SUT protein in the scutellar epidermal cell layer suggests the possibility of active transport of sucrose, and later maltose, into the scutellum, in addition to possible uptake of glucose mediated by hexose transporters. TaSUT1 was also found to localize to the scutellar ground tissue, suggesting a role in transport of the sucrose to the scutellar vasculature for loading into the phloem. In addition TaSUT1 was found in the aleurone cell layer, although its role in this cell type is unclear.

Localization of the OsSUT1 protein has not been reported in germinating rice seedlings. However, Matsukura et al. (2000) showed, by in situ hybridization, that the OsSUT1 transcript was present only in the vascular bundle of the scutellum of germinating rice seeds and localized specifically to companion cells of the leaf sheath of 5-d-old shoots. In the same study, cells surrounding the vascular bundle were shown to accumulate starch transiently, and it was suggested that these transient deposits may subsequently be remobilized back to sucrose which is then loaded into the phloem, via OsSUT1, for transport to the growing shoot. It was also suggested that initial uptake of sugars from the endosperm across the scutellar epithelium was in the form of glucose, and may be mediated by monosaccharide transporters. As yet no monosaccharide transporters have been reported in the literature to be expressed in the scutellum of germinating rice grains.

In the present work, the role that OsSUT1 plays in facilitating the transport of remobilized carbon reserves to the actively developing and growing sink tissues have been examined in detail. Analysis of sugar content, OsSUT1 expression data, OsSUT1 promoter::GUS expression analysis, and immunological localization of the OsSUT1 protein were carried out. This has allowed the function of OsSUT1 to be compared with that of the wheat orthologue TaSUT1 (Aoki et al., 2002) during germination of wheat seed.

Materials and methods

Plants and growth conditions

Oryza sativa L. cv. Nipponbare was used either untransformed or from T1 seed harvested from the OsSUT1 promoter::GUS T0 regenerated lines. Plants were grown to maturity in flooded paddy tanks in a greenhouse, in 75% compost/25% perlite, supplemented with Osmocote slow-release fertilizer. Growth was under natural light with a daytime temperature of 28 °C and a night-time temperature of 25 °C. T2 seeds were collected from the transgenic plants for use in germination experiments. De-husked rice seeds were subjected to heat-treatment at 50 °C for 5 d, prior to setting up germination experiments, in order to eliminate any residual dormancy effects and to obtain a more uniform germination rate (Zhang et al., 2005). For germination experiments, seeds were surface-sterilized by treatment with 70% ethanol for 1 min, washed twice in sterile distilled water, incubated in 50% (v/v) bleach in sterile distilled water for 15 min and, finally, washed thoroughly in eight changes of sterile distilled water. The sterilized seeds were plated out onto three layers of Whatman No. 1 filter paper circles and soaked in sterile distilled water in either 90 mm or 140 mm Petri dishes. The dishes were placed in a foil-covered box and placed in a growth room with a constant temperature of 25 °C.

Analysis of soluble carbohydrates

Seeds (25 per time point) were germinated as above and harvested at t=1, 3, 5, and 7 d post-imbibition (dpi). For the time points 1 dpi and 3 dpi, the germinating seeds were dissected into embryo and...
endosperm portions, with the former being rinsed in sterile distilled water on collection. Where present, coleoptiles and primary roots from the 3 dpi time point were excised and discarded. For the time points 5 dpi and 7 dpi the seedlings were divided into embryo, endosperm, coleoptile, and root sections. Samples were frozen in liquid nitrogen and then stored at -80°C until the extractions were carried out.

For extractions the samples were divided into three portions and each portion was weighed. The samples were plunged into a 5 ml volume of 80% ethanol at 85°C in which they were incubated for 10 min. The samples were then cooled at 4°C prior to grinding in a mortar and pestle with a further 5 ml of 80% ethanol being added during the grinding. Ground samples were quantitatively transferred to tubes with a further 5 ml of 80% ethanol. The tubes were centrifuged at 1620 g for 10 min at 4°C and the supernatants were carefully removed to fresh tubes, dried by rotary evaporation, and then taken up in known volumes of distilled water. High performance anion exchange chromatography (DX-600; Dionex, Sunnyvale, CA, USA) with pulsed amperometric detection (HPAEC-PAD) was used to separate and quantify the glucose, fructose, sucrose, and maltose contents of each sample using a Carbopac PA100 column eluted with a sodium acetate gradient in 100 mM NaOH.

RNA extraction and real-time PCR
For gene expression analysis, rice seeds were soaked with 5% sodium hypochlorite solution for 5 min, thoroughly washed with sterilized distilled water, placed on moist paper towels, and then germinated in darkness at 25°C. Samples were harvested daily up to 7 dpi. The samples from 3 dpi and later were separated into shoots, seeds, and roots. All samples were immediately frozen in liquid nitrogen and stored at -80°C until use. Each sample was ground to a fine powder in liquid nitrogen distilled water, placed on moist paper towels, and then germinated in For gene expression analysis, rice seeds were soaked with 5% sodium acetate gradient in 100 mM NaOH.

Preparation of an OsSUT1 promoter::GUS construct and transformation into rice
A translational fusion of the OsSUT1 gene promoter with the β-glucuronidase (GUS) reporter gene in a rice transformation vector was designed as follows. To obtain the OsSUT1 promoter::GUS construct, a DNA fragment spanning nucleotides –1675 to +19 of the translation start of OsSUT1 was amplified by PCR and then ligated into a transformation vector, pHZ12. This vector is a modification of pPZP202 (Hajdukiewicz et al., 1994) carrying hygromycin resistance. In this construct, the fusion protein was translated from the OsSUT1 start codon and the first six amino acids sequence of OsSUT1 is followed by the GUS sequence. The construct was introduced into Agrobacterium tumefaciens strain EHA101 by electroporation. The transformation of this construct into rice tissue (Oryza sativa L. cv. Nipponbare) was performed by the method of Hei et al. (1994).

GUS staining of transgenic plants
Whole seedlings sampled at 0, 1, 3, 5, and 7 dpi were stained for GUS expression in X-gluc solution at 37°C in the dark for either 5 h or overnight. The seedlings were submerged in X-gluc solution, containing 50 mM Na3PO4, 30 mM NaH2PO4, 0.4 M K2[Fe(CN)6], 0.4 M K4[Fe(CN)6], 3H2O, 10 mM Na-EDTA, 0.1% Triton X-100, 5 ml methanol, and 2 mM 5-bromo-4-chloro-3-indolyl-glucuronide cyclohexylamine salt, and were given a mild vacuum treatment, five 30 s periods, with vacuum released between each period, to aid infiltration of the X-gluc solution into the tissues. Post-staining treatment consisted of washing the seedlings twice in 25 mM Na-phosphate buffer (pH 7.2), followed by dehydration of the samples through a graded ethanol series to 70% ethanol. The whole seedlings were examined using either a Leitz M8 or Leica MZ7.5 stereo microscope and representative images were recorded using a Colorview SIS digital camera system.

Representative GUS-stained seedlings from the germination trial were embedded in LR White resin. Thin sections of between 0.5 and 1.5 μm in thickness were cut using an ultramicrotome and were collected onto glass slides. For visualization of the GUS expression, the sections were examined under dark-field illumination using a Leica DMR microscope and representative images were recorded using the Leica DC500 digital camera.

Embedding of germinating rice tissues in paraffin wax
Seed, coleoptile, and root tissues from rice seedlings were sampled at 0, 1, 3, 5, and 7 dpi, and fixed in 4% (w/v) formaldehyde solution in phosphate-buffered saline (130 mM NaCl, 7 mM Na2HPO4, 3 mM NaH2PO4, and 3 mM NaHPO4·H2O, pH 7.4) for 3 h at room temperature followed by incubation in fresh cold fixative solution for 24 h at 4°C. Seed were cut in half either transversely or longitudinally prior to fixation in order to facilitate penetration of the fixative solution. For the 5 dpi and 7 dpi time points the coleoptiles and roots were carefully cut off the seeds, as close as possible to their emergence point from the seed and were fixed separately. All the tissue samples were subjected to a gentle vacuum treatment, six 30 s intervals under vacuum, in the fixative solution to improve penetration of the fixative into the tissue. Post-fixation the samples were briefly washed twice in saline solution and were then dehydrated through a graded ethanol series. The samples were passed through a graded Histoclear:ethanol series prior to infiltration and embedding in paraffin wax.

Immunolabelling of OsSUT1 in tissue sections
Transverse sections, 12.5 μm in thickness, were cut using a Microm HM350 microscope and collected onto drops of distilled water on polystyrene-coated glass slides. The sections were baked onto the slides on a hotplate at 42°C for 24 h. The sections were treated with Histoclear to remove the wax, re-hydrated through a graded ethanol series, and washed in Digma buffer I (100 mM TRIS-HCl, pH 7.5, 150 mM NaCl). Next the sections were incubated in a solution of 0.5% (w/v) blocking powder (Roche) in Digma buffer I for 75 min

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followed by incubation in a blocking solution consisting of DIG buffer I containing 0.1% (v/v) polyoxyethylene sorbitan monolaurate (Twee 20) and 1% (w/v) BSA (Fraction V, Sigma) for 90 min. The sections were probed with an anti-OsSUT1 antibody (see Table 1 and Furbank et al., 2001), used directly without purification, at a dilution of 1 in 200 in blocking solution for 20 h at 4 °C. A preimmune serum was used, at a similar dilution, for the control. Following incubation in the anti-sera, the sections were washed for 3×15 min in blocking solution and were then treated with a secondary antibody anti-rabbit IgG (FC)-alkaline phosphatase conjugate (Promega), diluted 1 in 1250 in blocking solution, for 2.5 h. The sections were then washed 3×15 min in blocking solution, 1×10 min in DIG I plus 0.1% (v/v) Twee 20, 1×10 min in DIG buffer I alone, and finally 1×10 min in DIG buffer V (100 mM TRIS-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂·6H₂O). The sections were incubated in substrate solution, consisting of DIG buffer V containing 0.25 mM nitrotetrazolium blue chloride (Sigma) and 0.25 mM 5-bromo-4-chloro-3-indolyl phosphate (Sigma), to allow colour development. In tissue samples where endogenous alkaline phosphatases were found to be active, levamisole (tetramisole hydrochloride; Sigma) was added to a final concentration of 10 mM to the development solution. The reaction was stopped by washing the slides in several changes of distilled water. For visualization, the sections were examined under bright-field illumination using a Leica DMR microscope and representative images were recorded using a Leica DC500 digital camera system. For starch localization, wax sections were de-waxed, rehydrated through a graded ethanol series, and then stained in a solution of 0.5% (w/v) iodine in 5% aqueous potassium iodide for 10 min prior to examination under bright-field illumination with a Leica DMR microscope.

Results

Germination and early seedling growth

Shoot and root length were recorded at intervals during germination and early seedling growth in the etiolation conditions used in this work (data not shown). Based on these observations, the time points 1, 3, 5, and 7 dpi were chosen for further analysis. At 1 dpi the rice seeds had swollen as a result of imbibition and the coleorhiza and epiblast had started to expand. By 3 dpi the coleorhiza and epiblast had expanded further and the tips of the coleoptile and primary root had emerged; at this stage the endosperm was still firm. By 5 dpi the endosperm had started to soften, the coleoptile had expanded close to its maximal length, and the first leaf had emerged from within the coleoptile. The primary root had expanded considerably and primordia for lateral roots had become visible on the primary roots in the region close to their emergence from the seed. At 7 dpi the second leaf had emerged in many of the seedlings. Lateral roots on the primary roots had extended whilst the primary root had also continued to extend. In addition, secondary roots had emerged from the coleorhiza, reaching a length of between 5 mm and 15 mm at this time point. At this stage the endosperm had continued to degrade and soften.

Soluble carbohydrate content analysis

The soluble carbohydrate content of various tissues from rice seedlings, harvested over a time-course of 1–7 dpi, was determined using HPAEC-PAD (Fig. 1). In the embryo/scutellum portion of the seed (Fig. 1A), glucose and fructose levels were found to remain fairly constant from 3 dpi. High levels of sucrose were detected in this tissue at 1 dpi, which tended to decline gradually from 3 dpi. No maltose was detected in this tissue. Initial levels of glucose were low in the endosperm (Fig. 1B), but started to increase significantly from 3 dpi and reached 50-fold the initial level by 7 dpi, whereas fructose was not detected in the endosperm samples until 7 dpi and then only at a low level. The sucrose level in the endosperm was approximately one-fifth of that seen in the embryo at 1 dpi and increased about 2-fold over the time-course but still remained lower than the embryo. In the endosperm portion, maltose was also detectable from 3 dpi. The low level present at 3 dpi increased 10-fold by 5 dpi and doubled again by 7 dpi. In addition to maltose, trace levels of malt-oligosaccharides were also detected in the endosperm samples. Comparison of the carbohydrate profiles for the endosperm samples against individual standards, glucosyl maltotriose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltohexaose, indicated that low levels were present (data not shown). Glucose levels were about 2-fold higher in shoots than roots (Fig. 1C, D) and appeared to remain relatively unchanged at 5 and 7 dpi. Fructose levels were higher than glucose in both shoots and roots at 5 dpi and decreased slightly by 7 dpi. In shoots and roots comparable levels of sucrose were detected at 5 dpi and the levels decreased slightly in both tissues by 7 dpi. Maltose was not detected in these tissues.

Starch localization in tissue sections

Sections of germinating rice seeds were de-waxed, rehydrated, and stained with iodine to determine starch localization in tissue sections.
grain localization (Fig. 2). Within the embryo, high densities of starch grains were present in the scutellar parenchyma cells proximal to the scutellar epithelium and surrounding the scutellar vasculature, and in both cases the density of grains decreased in a gradient as distance from the epithelium and the vasculature increased. In addition, the starch grains extended to surround the vasculature where it divides into the coleoptile and primary leaf vasculature, and were present at high density at the base of the primary leaf shoots. Starch grains were also present in parenchyma cells surrounding the radicle. By 7 dpi the density of starch grains appeared to have decreased when compared with sections at a comparable level from a 5 dpi seedling (data not shown). This is presumably the result of the starch being remobilized to provide sucrose for uptake into the vasculature. By 7 dpi the first and second leaves had emerged and the seedling would presumably be commencing the transition from heterotrophic to autotrophic assimilate supply. Sections of embryos at earlier stages of development including 0 and 1 dpi were also stained for starch localization (data not shown). High densities of starch grains were found to be present in these samples, distributed throughout the scutellar parenchyma cells, as described for the 5 dpi sample above.

RT-PCR analysis of OsSUT1 expression

The level of expression of the OsSUT1 gene in germinating seedlings over a time-course post-imbibition was determined by RT-PCR (Fig. 3). In the seed fraction, comprising both the endosperm and the embryo, an initial peak of OsSUT1 transcript appeared at 2 dpi, after which the expression level declined until 5 dpi, and then it increased again. Shoots had emerged by 3 dpi, and from that point onwards the OsSUT1 expression appeared to increase in this tissue over 3 d, prior to a slight decrease on day 7 post-imbibition. In roots, OsSUT1 expression also increased from day 3 when the primary root emerged. However, the rate of increase of OsSUT1 expression in roots was lower than in shoots and it continued to increase at 7 dpi.

OsSUT1 promoter::GUS expression

T2 seed for two independent lines carrying the OsSUT1 promoter::GUS construct were sown, germinated, and harvested over a time-course up to 7 dpi and assayed for GUS expression (Fig. 4a). Expression was first detectable at 1 dpi and was confined to the embryo region of the seed. By 3 dpi the primary root and coleoptile had emerged from the germinating seeds and GUS expression was detected in both these organs as well as within the embryo portion of the seed. GUS staining was consistently more intense at the tip of the coleoptiles compared with the base. At 5 dpi both the root and coleoptile had extended in length and GUS expression was observed in both organs along with the seed. In some cases the coleoptile had split open to allow the first leaf to emerge and GUS expression was also apparent in the first leaf. In addition, lateral root primordia were apparent on the primary root around which there appeared to be GUS expression (data not shown). At 7 dpi there was GUS

Fig. 1. Analysis of soluble carbohydrates. The carbohydrate (glucose, fructose, sucrose, and maltose) content of (A) the embryo/scutellum and (B) the endosperm at 1, 3, 5, and 7 dpi, and (C) shoots and (D) roots at 5 dpi and 7 dpi were determined by HPAEC analysis. Results, expressed in µmol g⁻¹ FW, are the mean of three biological replicates and error bars show the standard error.
expression in the newly emerged first and second leaf blades, as well as in the coleoptile and embryo. Results were consistent between seedlings from both lines at the same time points. Untransformed control seedlings did not develop any blue colouration.

A coleoptile from a seedling at 7 dpi expressing GUS was embedded in resin and serial sections were examined under dark-field illumination (Fig. 4b). GUS expression was present within the two main vascular bundles of the coleoptile, and also in each vascular bundle of the first and second leaf throughout the entire length of the coleoptile and leaves (Fig. 4b, A–D). Expression, as determined by density of GUS crystals, was highest in the coleoptile and the first leaf, but low in the second leaf compared with the first. Close to the base of the coleoptile and leaves GUS expression appeared to be confined to the vasculature (Fig. 4b, A). However, with progression towards the tip (Fig. 4b, B–D) the expression pattern was more diffuse in the coleoptile and first leaf, with a gradient of GUS crystals in tissue surrounding the vasculature, decreasing in density away from the vasculature. The magnitude of signal along this gradient consistently increased closer to the tip of the coleoptile.

In sections through the embryo (Fig. 4c), GUS expression was absent from both the scutellar epithelial and adjoining parenchyma cell layers. GUS expression was present in the scutellar vasculature and could be traced through to the shoot vasculature. However, the expression was not specific to the phloem; there appeared to be GUS product outside the phloem, indicated by the presence of GUS crystals at a lower density in cells immediately surrounding it, which gradually declined as distance from the phloem increased. In roots, GUS expression was confined to the stele and was also observed in border cells at the root cap (data not shown).

**Immunolocalization of OsSUT1 in the scutellum**

Figure 5 shows typical transverse sections through a germinating rice seed in the region of the endosperm, scutellar epithelial cell layer, and scutellar parenchyma at 7 dpi. Whilst signal was detected in other regions of the scutellum, as indicated by the blue staining in the vascular tissue, the OsSUT1 protein was not detected in either the scutellar epithelial cells or the scutellar parenchyma cells, along the transport pathway from the endosperm. Similar results were obtained in sequential sections and in repeated labelling experiments for sections at both this time point and for the other time points examined in this study (data not shown). OsSUT1 was not detected in the aleurone cell layer at any of the time points examined (data not shown).
Fig. 4. (a) Expression of the OsSUT1 promoter::GUS construct in germinating seedlings. Whole seedlings from OsSUT1 promoter::GUS lines 1 and 120 were harvested at 1, 3, 5, and 7 dpi, and were assayed for GUS expression. (A–D) GUS expression in seedlings from line 1; (E–H) seedlings from line 120. (b) A series of transverse sections through a shoot from an OsSUT1 promoter::GUS line 1 seedling. A coleoptile from a 7 dpi seedling was embedded and serially sectioned along its length. The sections were viewed under dark-field illumination; GUS expression was apparent, by birefringence, as red crystals. (A) The coleoptile and emerged first and second leaves at a point close to the seed; note the zone of abscission in the coleoptile from which the whorled first and second leaves emerge. (B) The region approximately one-third distance from the seed, (C) at a distance of two-thirds from the seed, and (D) the tip of the coleoptile and emerged first and second leaf blades. c, Coleoptile; flb, first leaf blade; slb, second leaf blade. Scale bars = 200 μm. (c) A transverse section through the scutellar region of the embryo from an OsSUT1 promoter::GUS line 1 seedling at 7 dpi. se, Scutellar epithelium; sp, scutellar parenchyma; svb, scutellar vascular bundle. Scale bar=200 μm.
OsSUT1 was expressed in the scutellar vasculature leading out of the scutellar parenchyma tissue towards the coleoptile and first and second leaves (Figs 5, 6). Labelling was present in the scutellar phloem at 3 dpi and absent from the control (Fig. 6A, B). Analysis of sequential sections probed with the anti-OsSUT1 antibody showed that OsSUT1 is present along the entire length of the scutellar phloem (data not shown). Similar results were observed in the scutellar phloem at the other time points (Fig. 6C, D shows sections at 7 dpi). At higher magnification, OsSUT1 was observed to be present in the membranes of the sieve tubes and in the companion cells of the scutellar phloem (Fig. 6F) and absent from the control (Fig. 6E). By examining serial sections, OsSUT1 could be traced along the scutellar phloem towards the coleoptile and first and second leaf shoots. As the scutellar phloem neared these tissues it divided and branched into two main vascular bundles in the coleoptile and to multiple strands within the first and second leaf shoots (Fig. 6G, H).

**Immunolocalization of OsSUT1 in the developing coleoptile, first and second leaf blades**

Serial sections were cut longitudinally in respect to the coleoptile from a 5 dpi embryo (Fig. 7A, B). OsSUT1 was localized to the phloem of the coleoptile. The labelling could be traced along the entire length of the coleoptile vasculature in sequential sections. Transverse sections cut along the entire length of the coleoptile and first and second leaf blades from a seedling at 5 dpi showed that, at a point close to their emergence from the embryo, OsSUT1 was confined to the phloem of the two main vascular bundles in the coleoptile and in the phloem of the vascular bundles of both the first and second leaf blades (Fig. 7D, F, H, J). Labelling was absent from the controls. The labelling observed in all three tissues was also present in sequential transverse sections along their entire length, from their point of emergence from the seed to their tips (data not shown).

**Immunolocalization of OsSUT1 in the radicle and primary root**

Examination of transverse sections of the primary radicle, in a region within the germinated embryo, at 7 dpi, showed that OsSUT1 was confined to the phloem where it was present in the sieve elements and companion cells of the radicle (Fig. 8). Similar results were obtained in sections of the primary root at various points along its entire length (data not shown).

**Discussion**

Rice, like other plants examined, contains a number of sucrose transporters encoded by a family of genes, but detailed knowledge of their location and role in seed germination is lacking. Previous work has shown that OsSUT1 is the most highly expressed member of the SUT gene family in germinating rice seedlings (Aoki et al., 2003). The present work provides evidence for the occurrence of the OsSUT1 protein throughout the rice scutellar vasculature as well as in the shoot and root phloem regions, and its absence from the scutellar epithelium.
Activity of the *OsSUT1* promoter was also shown largely to correlate with the *OsSUT1* protein localization. In addition, these findings have implicated significant differences between rice and wheat in respect to location of orthologous SUT1 proteins, suggesting that sugar uptake may occur by different mechanisms along the transport pathways in germinating rice and wheat seedlings. These differences may be related to the different sugars which are produced as a result of starch breakdown in these respective tissues.

The sugar analysis and starch localization data obtained here are in agreement with, and expand upon, previously published work examining the sugar and starch contents of germinating rice seed (Murata *et al.*, 1968; Nomura *et al.*, 1969; Palmiano and Juliano, 1972; Matsukura *et al.*, 2000).

In the endosperm, sucrose predominates shortly after imbibition, which is similar to results from wheat seedlings.
However, there are some striking differences between rice and wheat. In rice, sucrose levels remain higher for a longer period post-imbibition, glucose levels increase more rapidly and to a much higher proportion of the total carbohydrate content, and maltose does not appear at a significant level until much later than in germinating wheat. Higher levels of glucose in the endosperm support the view that this may be the form in which carbohydrate is preferentially taken up across the scutellar epithelium in rice. Sucrose is probably synthesized from hexoses, which are transported across the scutellum, and is remobilized from starch grains in the cells surrounding the scutellar vasculature and at the base of the shoot, which start to break down at approximately 5 dpi. The high levels of sucrose observed in the scutellum, together with lower levels in the shoot and root, would provide a concentration gradient for sucrose loading in the rice scutellar vasculature. The lower levels of sucrose and high levels of hexoses in the shoot and roots indicate that sucrose is not accumulating in these tissues, but rather is being cleaved to hexoses for use in metabolism. Interestingly, fructose predominates over glucose in rice roots and shoots, whereas glucose is predominant in wheat roots and shoots albeit at an earlier time point to those used here (Aoki et al., 2006).

The OsSUT1 transcript was expressed in germinating seeds and, if translated, may result in some antibody binding. Analysis of expression of the OsSUT1 promoter::GUS construct and the OsSUT1 protein by immunolocalization gave consistent results, with the absence of both promoter activity and protein expression in scutellar epithelial and parenchyma cells and expression of both in the scutellar and shoot vasculature. The main difference observed between the expression of the OsSUT1 promoter and the OsSUT1 protein was that the latter was confined solely to the phloem, whereas GUS expression was sometimes detected in the surrounding tissues. This could either be due to movement of the GUS protein or product before crystallization, or represent different patterns of localization of the OsSUT1 transcript versus the protein. Although post-transcriptional regulation of OsSUT1 protein abundance is possible, such large discrepancies have not been observed in previous in situ localization work in rice (Furbank et al., 2001). Since the GUS protein is 68 kDa in size and is functional in a tetrameric form (Jefferson et al., 1987), it seems unlikely that it would be able to move via symplastic connections. However, Fukuda et al. (2005) examined the distribution of proteins, including GUS, in the companion cells and sieve elements in rice. These authors presented evidence to suggest that the GUS protein, under control of a companion cell-specific promoter, could enter sieve elements and xylem parenchyma cells, and speculated that such movement may be mediated by protein unfolding. Alternatively, it may be the product of the GUS-mediated reaction that is able to move via plasmodesmatal connections before crystallization.

In this context, it is interesting to observe that the GUS product appeared outside the phloem to a greater degree closer to the tips of the coleoptile and first leaf blade than at the base of these structures. This suggests that the GUS product may have moved symplastically in this region of these tissues. This possibility is supported by evidence from Haupt et al. (2001), who demonstrated symplastic phloem unloading in sink leaves of young barley plants.
Towards the base of the coleoptile and primary leaves, where the GUS expression is more confined to the phloem, there may perhaps be fewer symplastic connections or the plasmodesmata may be closed to prevent sucrose from leaking out into areas where it is not required for metabolism. The companion cell/sieve element complexes in mature transport phloem of Arabidopsis roots were found to be symplastically isolated from the surrounding parenchyma, whereas in the elongating zone towards the tip of the main and lateral roots symplastic transport routes to the parenchyma existed (Oparka et al., 1994, 1995). Evidence for the sparsity of plasmodesmata in transport phloem was extended in studies by Kempers et al. (1998), and van Bel (2003) and the references therein. In the current work it is unlikely that the primary leaves have undergone the sink to source transition, with its accompanying decrease in plasmodesmatal permeability which limits the symplastic continuity between the companion cell/sieve element complexes and surrounding parenchyma cells (Roberts et al., 1997; Oparka et al., 1999). This work suggests that a major difference exists between rice and wheat in the transport mechanism for uptake of remobilized carbohydrate reserves across the scutellum from the endosperm. In wheat, it was suggested that TaSUT1, as well as a hexose transporter, is involved in transport of sugars across the scutellar epithelial cell layer, facilitating sucrose and possibly maltose to be taken up (Aoki et al., 2006). From the present work, however, it appears that the rice homologue OsSUT1 does not perform a similar function. Rather, the evidence supports a model that glucose is the form in which sugars are predominantly taken up across this symplastic discontinuity in rice. Exactly how glucose is taken up across the scutellar epithelium is still to be determined; as yet no hexose transporters with this function in rice have been reported in the literature. Another major difference between rice and wheat is the apparent lack of OsSUT1 in the aleurone layer, whereas TaSUT1 was found to be expressed in the wheat aleurone cell layer post-imbibition (Aoki et al., 2006). The role that TaSUT1 plays in the wheat aleurone is unclear, although it has been speculated that it may act as an effluxor to export sucrose to the endosperm at early post-imbibition. Chrisepeels et al. (1973) suggested that, in barley, the aleurone layer may continuously synthesize and secrete sucrose. The lack of OsSUT1 labelling in the aleurone observed in this work may indicate that sucrose is not present at significant levels in rice aleurone cells during rice germination, or that OsSUT1 is unable to act as an effluxor. Alternatively it may be the case that another member of the OsSUT family such as OsSUT3, rather than OsSUT1, plays a role in the aleurone but it is not detected by the antibody.

The correlation of expression of the OsSUT1 promoter and OsSUT1 protein in the scutellar vasculature, from this work, suggests that the major function of OsSUT1 is in phloem loading of the sucrose that is synthesized in the scutellum, and subsequent retrieval along the long-distance transport pathway. The scutellum has previously been reported as a site for sucrose synthesis in germinating rice seed. Nomura and Akazawa (1973, 1974) and Chávez-Bárcenas et al. (2000) have shown that SPS is located in the scutellum and is active during germination and early growth of rice seedlings. Sucrose may be synthesized directly from the glucose that is transported across the scutellar epithelium, and also from degradation of starch granules that are found at highest density surrounding the scutellar vasculature. Furthermore the presence of starch grains in parenchyma cells alongside the entire length of the scutellar vasculature, and surrounding the differentiated shoot vasculature, suggests that loading of remobilized carbohydrate may occur at any point along the vasculature, and may not be limited to just the endosperm end of the scutellar phloem. The presence of high densities of starch grains present in the embryo at 0 dpi and 1 dpi indicates that the embryo already contains substantial starch reserves before germination occurs. Presumably there is uptake of sugars into the scutellar parenchyma cells while the embryo is still developing, during the grain-filling period, and this sugar is stored in the form of starch for remobilization to sugars on germination. At this stage it is unknown as to whether any members of the SUT family are involved in sugar transport into the embryo during the grain-filling stage of development.

In the current work it was observed that the OsSUT1 protein was present in both the companion cells and in the sieve elements. Although there are few data in the literature on the cellular localization of SUT transcript and protein in cereals species, Matsukura et al. (2000) reported that localization of the OsSUT1 transcript is companion-cell specific. From previous work in phloem of both wheat vegetative tissues (Aoki et al., 2004) and germinating wheat seeds (Aoki et al., 2006) it was found that SUT1 transcript was present only in companion cells but SUT protein was present only in sieve elements. This supports the hypothesis that the OsSUT1 protein may be imported to the sieve elements from adjacent companion cells via plasmodesmatal connections, but the differences between rice and wheat suggest that there may also be interspecific variation in this mechanism in cereals as has been observed in dicotyledons. The site of synthesis and cellular location of SUT proteins in the phloem sieve element/companion cell complex in dicotyledons has been the subject of debate (Lalonde et al., 1999; Oparka and Turgeon, 1999). In the Solanaceae, SUT1 protein appears to be confined to the plasma membrane of the sieve element while the transcript is localized to the companion cells (Kühn et al., 1997). By contrast, in Plantago major and Arabidopsis the SUC2 protein is present in both cell types (Stadler et al., 1995; Stadler and Sauer, 1996). The absence of SUT transcript from the sieve element is not
surprising, considering the lack of functional nuclei in mature sieve elements (van Bel and Knoblauch, 2000, and references therein). The presence of SUT protein in both cell types does, however, have bearing on the issue of whether SUT transcript is transported symplastically into the sieve elements from the companion cells and translated in this cell type or whether the protein itself is moved from the companion cell to the sieve element. Both these models have been proposed and evidence for both presented in the literature (Stadler et al., 1995; Stadler and Sauer, 1996; Kühn et al., 1997; Lalonde et al., 2003).

In summary, in contrast to germinating wheat seeds, OsSUT1 in rice does not appear to be involved in the transport of sugars from the endosperm to the embryo. Instead it is proposed that this transporter loads sucrose synthesized in the scutellum into the sieve elements and serves to retrieve sucrose from the phloem apoplasm along the transport pathway to the shoot and root.

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References


