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Cell Wall Invertase in Developing Rice Caryopsis: Molecular Cloning of *OsCIN1* and Analysis of its Expression in Relation to its Role in Grain Filling

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To establish the significance of cell wall invertase in grain filling of rice (*Oryza sativa* L.), we cloned a cDNA for a cell wall invertase from developing grains of rice. The cDNA, designated *OsCIN1*, contains an open reading frame of 1731 bp encoding a polypeptide of 577 amino acid residues. The deduced amino acid sequence showed typical features of the cell wall invertases, including a β-fructosidase motif and a cysteine catalytic site, and shared 78.6 and 73.7% identity with maize cell wall invertases, Incw1 and Incw2, respectively. *OsCIN1* is expressed in roots, in sink- and source-leaves, and in panicles. During the course of grain filling in the caryopses, *OsCIN1* transcript is detectable only in the very early stage of their development, 1–4 d after flowering, when the cell wall invertase activity is the highest and the increase in caryopsis length is rapid. In situ localization of the mRNA revealed that *OsCIN1* is expressed preferentially in the vascular parenchyma of the dorsal vein, integument and its surrounding cells, and is expressed weakly in the nucellar projection and nucellar epidermis. These results suggest that, during the early stage of caryopsis development, *OsCIN1* is important for supplying a carbon source to developing filial tissues by cleaving unloaded sucrose in the apoplast.

Key words: Cell wall invertase (EC 3.2.1.26) — Grain filling — *Oryza sativa* L. — Sucrose transport.

Abbreviations: CWI, cell wall invertase; DAF, days after flowering; DIG, digoxigenin; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR.

The nucleotide sequence reported in this paper has been submitted to the GenBank, EMBL under accession number AB073749.

Introduction

Invertase (β-fructofuranosidase; EC 3.2.1.26) catalyzes the hydrolysis of sucrose to glucose and fructose. In higher plants, invertases have been classified into three isoforms based on the optimum pH of their activity (see Sturm 1999 for review). Neutral and alkaline invertases most probably exist in the cytosol, but are not well characterized. Acid invertases are further classified into soluble and insoluble forms. Soluble acid invertase exists in the vacuole and insoluble acid invertase is present in the apoplast and is usually ionically bound to the cell wall. Recently a novel type of apoplastic invertase, which is not bound to the cell wall, has also been reported (Kim et al. 2000).

Among the invertase isoforms, insoluble acid invertase (cell wall invertase, CWI) is considered to be one of the key enzymes involved in establishing sink-strength in various sink tissues. A maize mutant, *miniature-1*, lacking CWI activity in the pedicel and endosperm, showed a marked inhibition of seed growth during early stages of development. Its final grain weight is decreased to just one-fifth of the wild-type grain weight (Miller and Chourey 1992). The *miniature-1* locus encodes a CWI, *Incw2*, which is expressed exclusively in the basal part of the endosperm at the cell division stage of development (Taliercio et al. 1999). The Incw2 protein, localized in the apoplast between basal endosperm and the pedicel, is necessary for normal development of the endosperm (Cheng et al. 1996). The authors concluded that the maintenance of a physiological gradient of sucrose between pedicel and endosperm, mediated by the Incw2 protein, is crucial for normal development of maize kernels.

In developing fava bean seeds at the pre-storage phase, high levels of hexose sugars in the cotyledons and the apoplastic endospermal space correlate with high levels of CWI activity in the seed coat, and CWI gene expression is confined to the innermost cell layer of thin-walled parenchyma and the chalazal vein (Weber et al. 1995). At the onset of the storage phase, CWI activity declines and an active sucrose uptake system is formed in the epidermis of the embryo. The role of CWI was also studied in transgenic carrot plants using antisense suppression technique. Transgenic plants with reduced activity of CWI could not develop taproots, whereas antisense suppression of sucrose synthase in carrot resulted in the formation of smaller roots and leaves with leaf to root dry weight ratio unchanged (Tang et al. 1999, Tang and Sturm 1999). These results collectively show that CWIs play important roles in differentiation and/or early development of sink organs rather than storage of assimilates in them.

The role of CWI in the development of cereal seeds has not been extensively studied except for the work in maize described above. Furthermore, it should be noted that seed morphology differs greatly among cereal species. For exam-

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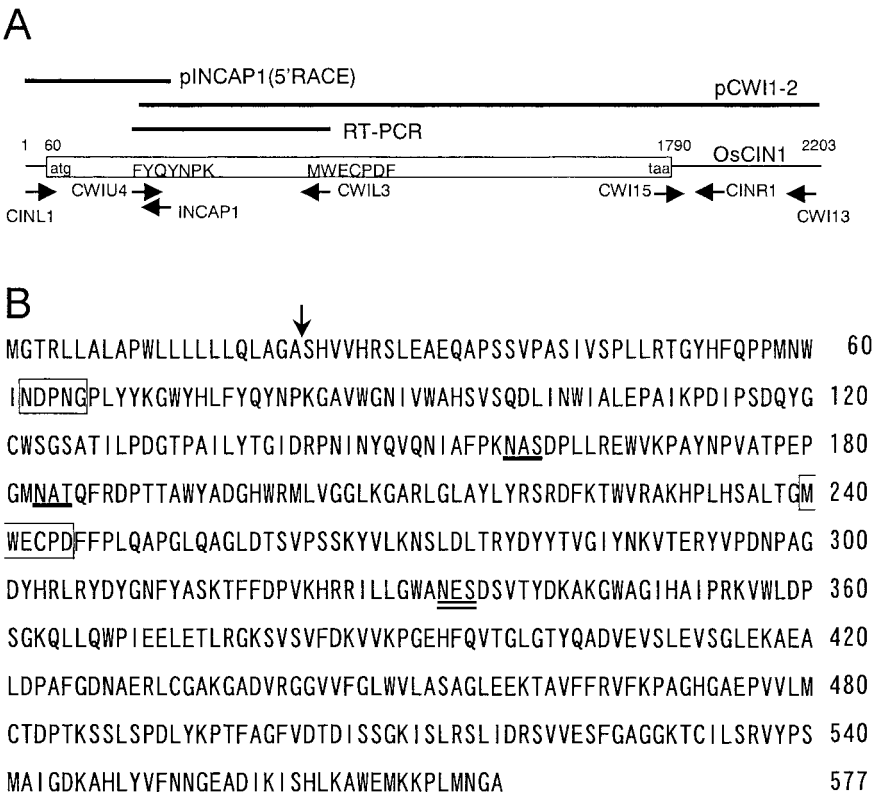

Fig. 1 Schematic diagram of the structure and the deduced amino acid sequence of OsCIN1. (A) Structure of *OsCIN1* cDNA and the cDNA fragments used to assemble the full-length sequence. The open box and the lines represent the coding region and untranslated sequence, respectively. The bold lines represent cDNA fragments derived from 5′RACE, library screening and RT-PCR. The positions of the PCR primers used are shown with arrows. (B) Deduced amino acid sequence of OsCIN1. Conserved β-fructosidase motif (NDPNG/A) and cysteine catalytic site (MWECP/V) are boxed. Potential N-glycosylation sites are underlined, and one conserved site is double underlined. Arrow indicates the potential signal peptide cleavage site.

ple, the importing vein extends along the length of caryopses in rice and wheat while it terminates in the pedicel parenchyma at the base of kernels in maize. The aleurone transfer cells, which may facilitate the transport of assimilates into endosperm, are present in maize, wheat and barley but are absent in rice (see Patrick and Offler 1995 for review). Additionally, rice seeds lack the endosperm cavity present in wheat and barley. With such marked morphological differences in seed structure between different cereal species, it is likely that different mechanisms for assimilate transport and metabolism occur in cereal seed development. In rice, the putative role of CWI in the molecular mechanism of grain filling has not been studied. In this study we have cloned the cDNA of a gene for CWI expressed in rice caryopsis, and have investigated its temporal and spatial expression during grain filling in relation to its roles in sucrose transport and metabolism. We compare its putative role in grain filling in relation to that of the rice sucrose transporter, *OsSUT1*.

Results

Cloning and sequence analysis of cDNA for rice CWI

Degenerate PCR primers were designed from conserved regions in the peptide sequences of known invertases from other plant species, and PCR was performed using the first strand cDNA from rice panicles at the early to mid-ripening stage as a template. Since the deduced amino acid sequence of an amplified cDNA fragment showed strong similarities to the corresponding regions of maize Incw1 and Incw2, it was used as a probe to screen a cDNA library from developing rice grains. Although sequence analysis of the longest positive clone revealed that it has strong similarities to the maize CWIs, it lacked the 5′ end of the cDNA. To obtain the sequence of 5′ region of the clone, we performed 5′ rapid amplification of cDNA ends PCR (5′RACE-PCR). The nucleotide sequence of the 3′ region of the resultant 5′RACE-PCR product completely matched the corresponding 5′ region of the cDNA clone obtained by library screening, indicating that the two cDNA fragments are derived from the same gene. This was finally confirmed by reverse transcription-PCR (RT-PCR) using a pair

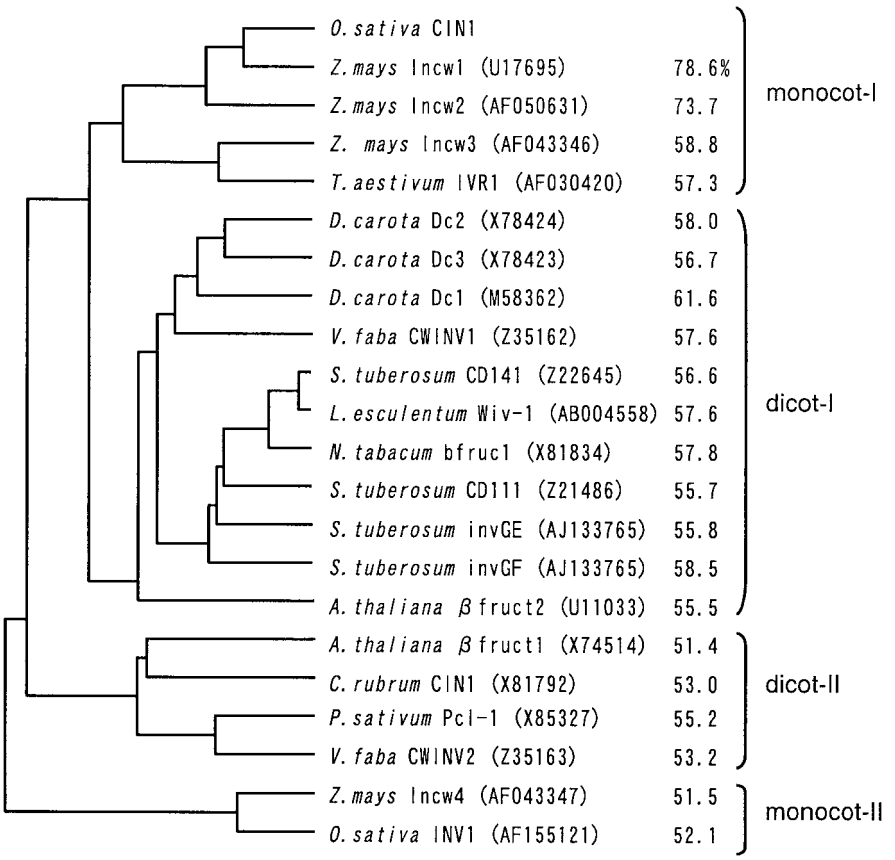

Fig. 2 A phylogenetic tree based on the amino acid sequences of the cell wall invertases. The identities of the amino acid sequences as compared to OsCIN1 protein are also shown.

of primers designed from the putative 5′- and 3′-non-coding sequence of the two cDNA fragments. We then designated the assembled sequence *OsCIN1* as a cDNA for a putative CWI gene (see Materials and Methods for details).

Fig. 1A illustrates the structure of the *OsCIN1* cDNA. The cDNA, total length 2,203 bp, contains one open reading frame starting at nucleotide 60 with an ATG start codon and ending at nucleotide 1,790 before TAA stop codon. There is an in-frame stop codon TAG upstream from the start codon at nucleotide 48, which confirms that this start codon is in fact the translation initiation in *OsCIN1*. The open reading frame encodes a polypeptide of 577 amino acid residues with a calculated molecular mass of 64.0 kDa and an isoelectric point of pI = 8.03. There is a conserved β-fructosidase motif, NDPNG/A, and a cysteine catalytic motif, MWECP/V (Fig. 1B). The fifth residue in the catalytic domain is proline as in the other known CWIs, whereas in vacuolar isoforms it is valine. There are three potential N-glycosylation sites (NXS/T), one of which is conserved in all plant CWIs identified so far (Fig. 1B). In addition, computer analysis of the deduced amino acid sequence predicted a potential cleavage site between amino acid residues 22 and 23, and extracellular localization of the protein (Fig. 1B). This sequence data is indicative of OsCIN1 being a CWI.

A phylogenetic tree based on the predicted amino acid sequences from known CWIs is shown in Fig. 2. The CWIs are classified into four groups, monocot-I, -II, dicot-I and -II. OsCIN1 belongs to the monocot-I group with maize and wheat CWIs. It is most closely related to Incw1 from maize with 78.6% amino acid identity. During the course of this study we found a novel genomic sequence for rice CWI named *INV1* (accession No. AF155121) in the database. The predicted amino acid sequence of INV1 shares only 52.1% identity with OsCIN1. The INV1 protein and maize Incw4 constitute the monocot-II group and are more distantly related to other CWIs in the phylogenetic tree. Kim et al. (2000) reported that the maize Incw4 protein is uniquely characterized by acidic pI, short C-terminal extension and modified motif for catalytic domain, and suggested that Incw4 is a unique type of CWI unbound in the apoplast. As the rice INV1 protein seemingly has characteristics similar to those of maize Incw4, it might be an unbound type CWI in rice.

Expression of OsCIN1 in different organs of rice

To investigate organ specificity of *OsCIN1* expression, we hybridized a Northern blot of RNAs isolated from different tissues of rice plants with a gene specific probe of *OsCIN1*.

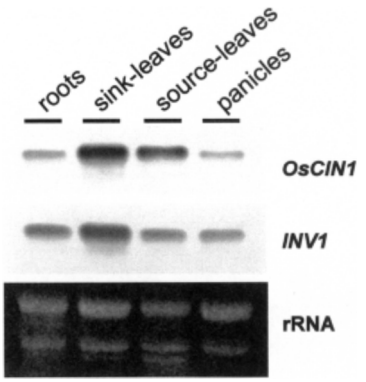

Fig. 3 Expression of the *OsCIN1* and *INV1* mRNA in various organs of rice plant. Total RNAs were isolated from the roots of rice plants 20 d after germination (roots), immature flag leaves covered with the leaf sheath (sink-leaves), mature flag leaves (source-leaves), and panicles of 5 d after flowering. Total RNA (5 μg) was loaded per lane, and the blots were hybridized with DIG-labeled riboprobes generated from 3′-non-coding region of either *OsCIN1* or *INV1* cDNA. The lower panel shows the ethidium bromide-stained rRNA.

OsCIN1 was expressed in all organs tested, i.e. roots, sink-leaves, source-leaves and panicles (Fig. 3). The size of the detected band estimated to be ca. 2 kbp is consistent with the cDNA size. The steady-state transcript levels were higher in sink- and source-leaves than in the other two tissues tested. Recently another putative CWI gene, *INV1*, has been appeared in the database as described above. To compare the expression pattern of *INV1* with *OsCIN1*, we hybridized a gene specific probe for *INV1* to the same blot. As in the case of *OsCIN1*, the *INV1* transcript was detected in all organs tested with the highest level in sink-leaves (Fig. 3).

OsCIN1 is expressed in the developing caryopsis in the early grain filling stage

As CWIs are reported to be important in sucrose metabolism in seed development, we further investigated the expression of *OsCIN1* during grain filling in the caryopsis. The amount of *OsCIN1* transcript in the caryopsis was abundant in the very early stage of grain filling, 1–3 d after flowering (DAF) (Fig. 4A). At this developmental stage the caryopsis was found to be rapidly elongating, but dry weight did not increase markedly (Fig. 4B). Interestingly, the *OsCIN1* expression level fell sharply to a negligible level thereafter; and the transcript level was very low even at 4 DAF (Fig. 4A). The activity of cell wall invertase was also high in the early developmental stage, but then declined to a low level (Fig. 4C). The levels of sucrose and hexoses (glucose plus fructose) were also high in the early stage of development, peaked at 3 DAF, and then declined in a similar way (Fig. 4D). The expression level of *INV1* was relatively constant from 1 to 7 DAF, and then declined to a low level (Fig. 4A).

Recently the rice sucrose transporter (*OsSUT1*) has been

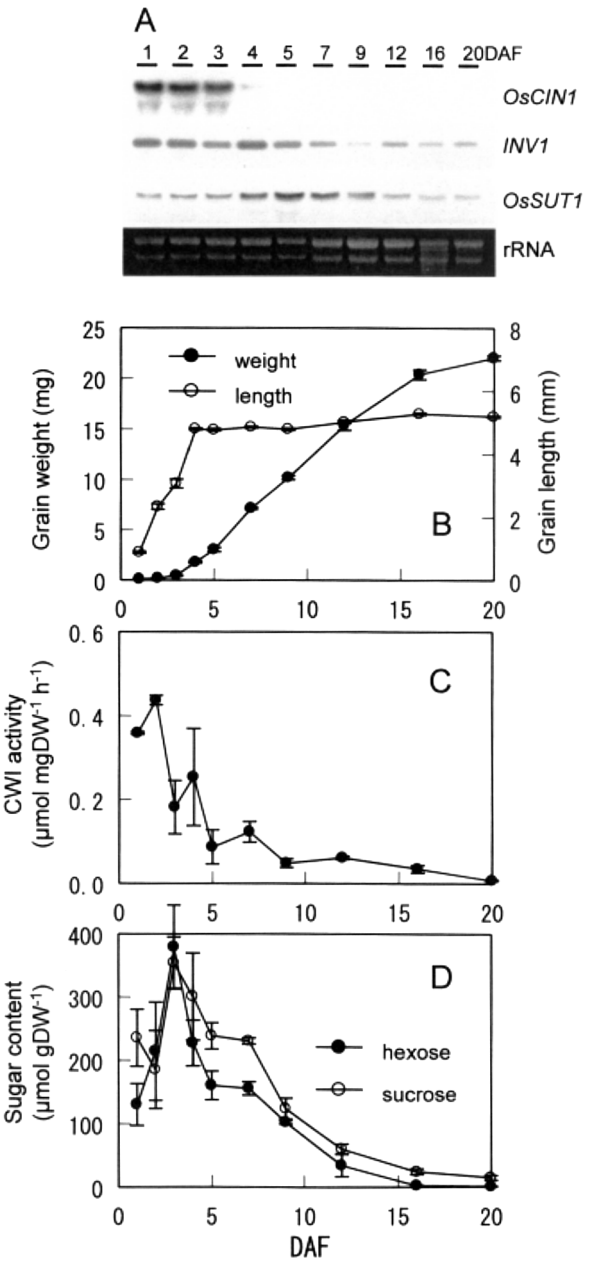

Fig. 4 Comparison of expression levels of *OsCIN1*, *INV1* and *OsSUT1*, and growth data from rice caryopsis. (A) Expression levels of *OsCIN1*, *INV1* and *OsSUT1* mRNA in caryopses during the grain filling. (B) Growth curves to show the weight and the length of developing rice grains. (C) Activity of cell wall invertase and (D) sucrose and hexose contents of developing rice caryopses. Values in panels B, C and D are the means ± SE of at least three replications.

shown to play an important role in rice caryopsis at the starch accumulating stage (Furbank et al. 2001, Scofield et al. 2002). To compare the *OsSUT1* with *OsCIN1* in light of their expression patterns in the developing rice caryopsis, the Northern blot for grain development was hybridized with an *OsSUT1* spe-

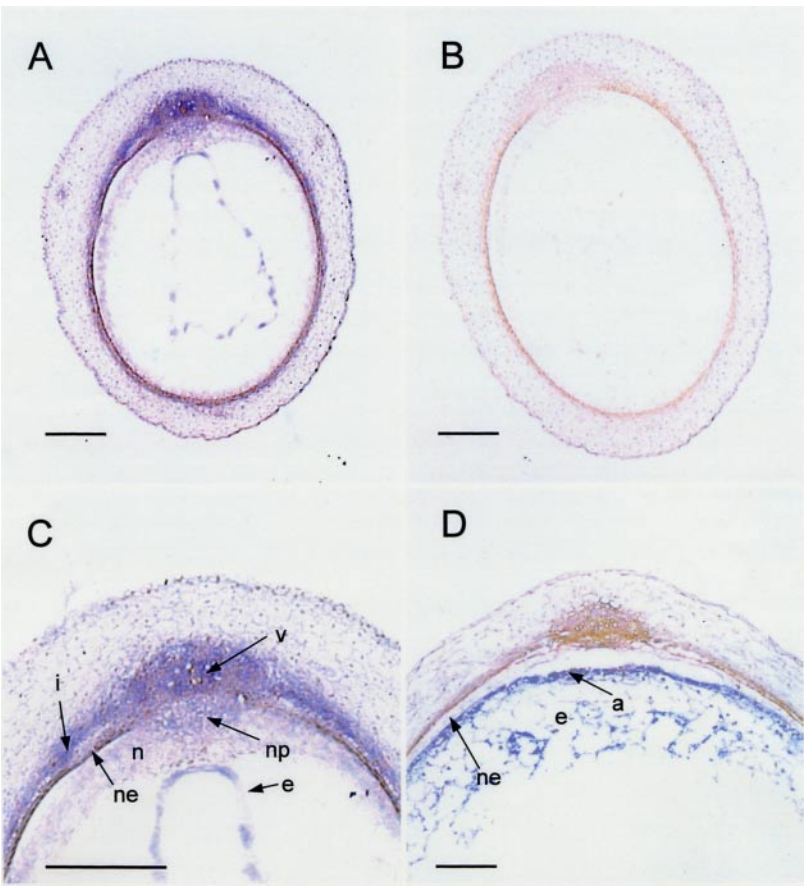

Fig. 5 In situ mRNA localization of *OsCIN1* and *OsSUT1* in transverse sections of developing rice caryopses. (A) Caryopsis at 1 DAF hybridized with an *OsCIN1* antisense riboprobe. (B) Caryopsis at 1 DAF hybridized with an *OsCIN1* sense riboprobe as a negative control. (C) The same section as A at higher magnification. (D) Caryopsis at 5 DAF hybridized with an *OsSUT1* antisense probe. a, aleurone; e, endosperm; i, integument; n, nucellus; ne, nucellar epidermis; np; nucellar projection; v, dorsal vein. Bar = 0.1 mm.

cific probe. The mRNA level of sucrose transporter *OsSUT1*, unlike that of *OsCIN1*, was low in the very early stage of grain filling and increased to a high level at 5–9 DAF (Fig. 4A), at which the caryopsis had ceased to elongate and had started to rapidly gain dry weight (Fig. 4B).

OsCIN1 mRNA in the developing caryopsis is localized mainly in the maternal tissues

Since *OsCIN1* was expressed in the developing caryopsis of the early grain filling stage, in situ localization of the mRNA was performed on rice caryopses at 1 DAF using a gene specific probe (Fig. 5A). At this developmental stage the endosperm, a filial tissue, is less developed, forming only a peripheral layer that tends to crumple during the sample preparation. *OsCIN1* mRNA was found to accumulate in the vascular parenchyma of the dorsal vein, the importing vein in the caryopsis, and the integument and its surrounding cells. In addition, at higher magnification (Fig. 5C), a weak signal was also observed in other maternal tissues, i.e. nucellar projection and nucellar epidermis. Occasionally a weak signal was also observed in the endosperm. No signal was detected in an equivalent section probed with the *OsCIN1* sense probe (Fig. 5B). In a section of a caryopsis at 5 DAF, no *OsCIN1* transcript was detected (data not shown); and, this correlates to the result of the Northern blot analysis shown in Fig. 4A. We localized the mRNA for the rice sucrose transporter, *OsSUT1*, in the section of 5 DAF when its mRNA level was shown to be highest (Fig. 4A). At this developmental stage, the endosperm has developed well and the aleurone layer surrounding the endosperm has been formed. The nucellus has been compressed by the developing endosperm and has almost been degenerated. The *OsSUT1* transcript was strongly detected in the aleurone and the endosperm (Fig. 5D).

Discussion

In this study, we have characterized a CWI gene from rice named *OsCIN1*. Analysis of the predicted amino acid sequence of OsCIN1 revealed that it possesses many features reported for other CWIs (Fig. 1). These include the two conserved

motifs representing a β-fructosidase and a catalytic domain, putative signal peptide probably required for extracellular localization of the protein and a basic pI that may be important for ionic binding with the cell wall component (Sturm and Chrispeels 1990).

Expression pattern analysis revealed that the *OsCIN1* transcript is present in roots, sink-leaves, source-leaves and panicles (Fig. 3), suggesting that *OsCIN1* has roles in both sink and source tissues. In source tissues, CWIs have attracted less attention. Although there are some instances of the expression of CWI genes in source tissues such as *Atbfruct1* in Arabidopsis (Tymowska-Lalanne and Kreis 1998) and *Wiv-1* in tomato (Ohyama et al. 1998), their roles are still unclear. Recently, some hypotheses for the potential roles of insoluble invertase in leaves, e.g. sucrose metabolism in epidermis and sugar sensing, were proposed by Kingston-Smith et al. (1999). Involvement of CWIs in stress responses such as wounding (Ohyama et al. 1998) and salinity (Fukushima et al. 2001) has been also reported. However, further investigation is obviously needed to understand the role(s) of *OsCIN1* in source leaves. In sink tissues, in contrast, expression of CWI genes has been well documented and possible involvement in the unloading of sucrose has been suggested (see Sturm 1999 for review). This could explain the expression of *OsCIN1* in roots, sink-leaves and panicles.

In developing rice caryopses, the *OsCIN1* transcript was detectable only in the very early stage of development, 1–4 DAF (Fig. 4A). The *OsCIN1* expression seemed to be associated with the activity of CWI and the hexose content, which were also higher in this phase than in the following starch accumulating stage (Fig. 4C, D). However, the remaining lower activity of CWI after 5 DAF implies a longer half-life of OsCIN1 protein than the corresponding mRNA and/or the existence of isogenes contributing to enzyme activity. At this early stage of caryopsis development, dry weight increase is still not prominent; however, the caryopsis length is rapidly increasing (Fig. 4B), which is known to reflect the rapid increase in the number of endosperm cells in rice (Hoshikawa 1993). These data suggest that *OsCIN1* is involved in the proliferation of endosperm cells rather than starch accumulation through sucrose metabolism. In developing maize kernels, a high level of CWI activity is reported to coincide with cell division in the endosperm (Cheng et al. 1996). Weber et al. (1996) have shown that both higher CWI activity and high hexose levels in cotyledons of *Vicia faba* are correlated with prolonged mitotic activity. Our results along with these data collectively suggest that the CWIs play an important role in developing seeds at the cell division stage in various plant species including rice caryopses.

We also investigated the mRNA level of *INV1*, a gene for a putative unbound type CWI. The organ specificity of *INV1* expression was largely similar to that of *OsCIN1* (Fig. 3). During the course of caryopsis development, in contrast to *OsCIN1*, the mRNA level of *INV1* was relatively constant from 1 to 7 DAF (Fig. 4A). However, the roles of the unbound type CWIs including INV1 remain quite unclear so far. Further studies are clearly needed to understand them.

In situ localization of mRNA in the caryopses of 1 DAF clearly showed that the *OsCIN1* transcript accumulates mainly in the maternal tissues, i.e. vascular parenchyma of the dorsal vein, integument and its surrounding cells (Fig. 5A, C). Expression of CWI in the early stages of seed development has been reported for maize *Incw2* encoded by *miniature-1* locus (Cheng et al. 1996). Incw2 is closely related to OsCIN1 in the phylogenetic tree, and is believed to play a crucial role in providing hexose sugars for the development of the endosperm in the cell division stage (Cheng and Chourey 1999), which seems to be similar to the role of *OsCIN1* suggested in this study. Interestingly, expression of *Incw2* is confined exclusively to a filial tissue, i.e. the basal part of the endosperm (Taliercio et al. 1999), while the expression of *OsCIN1* was weak and occasional in endosperm, but strong in maternal tissues. Further investigation including protein and/or activity localization is required to clarify this point.

The localization of mRNA for a CWI in maternal tissues of developing seeds has previously been reported in fava bean with expression of *VfCWINV1*, which is confined to the chalazal vein and the thin-walled parenchyma of seed coat during pre-storage phase (Weber et al. 1995). The thin-walled parenchyma cells of legume seeds are known to be the efflux site of assimilates (Patrick and Offler 1995), and VfCWINV1 is believed to cleave the unloaded sucrose (Weber et al. 1995). This cleavage of sucrose in the apoplast is thought to increase the sucrose concentration gradient between cells of thin-walled parenchyma and the apoplastic space, and thus to facilitate post-phloem sucrose transport in the young seeds. Similar expression of *OsCIN1* in the maternal tissues observed in this study suggests that these tissues could be a potential candidate(s) for the sucrose efflux site, and that OsCIN1 may play a role in the mechanism of sucrose efflux by cleaving the released sucrose in the extracellular space, although the route of assimilate transport in rice caryopses at the early stage of grain filling has not yet been clarified.

In the later stage of grain filling, Oparka and Gates (1981a), Oparka and Gates (1981b) identified the nucellar epidermis surrounding the aleurone layer as a site of sucrose efflux from maternal tissues and suggested that the aleurone layer takes up the released sucrose. They observed symplastic continuity from the dorsal vein phloem to the nucellar epidermis through a “pigment strand” a specialized parenchyma having the suberized cell wall, which could confine the movement of assimilates into the symplast. However, the suberization of the cell wall occurs only after the cessation of caryopsis elongation (Oparka and Gates 1982), suggesting that the site of sucrose efflux during the early grain filling stage is not necessarily restricted to nucellar epidermis, and that the major route of sucrose transport from the dorsal-vein phloem to the efflux site could differ depending on the developmental stage of cary-

opsis. Further studies using fluorescent dyes are required to elucidate this point.

We also observed here that *OsSUT1* transcript was highly expressed in the aleurone and endosperm of caryopses at 5 DAF, the early starch accumulating stage (Fig. 5D). Similar localization of OsSUT1 protein and mRNA at this developmental stage has recently been reported (Furbank et al. 2001). These results are consistent with the above-mentioned hypothesis on the route of assimilate transport into filling rice grains (Oparka and Gates 1981a, Oparka and Gates 1981b). Northern blot analysis clearly showed that high expression levels of *OsSUT1* occurred after *OsCIN1* expression and CWI activity had significantly declined (Fig. 4A). There may be two modes of sucrose supply to the endosperm tissues at different developmental stages in rice caryopses. CWI-mediated hexose supply occurs at the cell division stage and direct sucrose uptake, mediated by *OsSUT1*, occurs at the starch accumulating stage. A similar observation has been shown in fava bean, where CWI activity declines at the onset of the storage phase and, concomitantly, active sucrose uptake by sucrose transporter into the embryo becomes predominant (Weber et al. 1997).

In conclusion the data presented here suggest that *OsCIN1*, the first CWI gene identified in rice, plays a role in the sucrose metabolism in the early stages of caryopsis development, in which rapid proliferation of endosperm cells occurs. Our results, together with those from legumes and maize, imply a universal importance of CWI in the early stage of seed development among plant species. However, the role(s) of *OsCIN1* in other tissues including source leaves remains unclear. Our ongoing study using antisense suppression of this gene may help to determine its role in source tissues.

Materials and Methods

Plant material

Rice plants (*Oryza sativa* L. cv. Nipponbare) were grown under field conditions in plastic pots filled with the soil from the paddy field of NARC, Joetsu, Japan. The plants were moved to the greenhouse just before heading to avoid cleistogamy often caused by rainfall, which makes it difficult to monitor the date of anthesis. At anthesis each caryopsis was marked and subsequently sampled following maturity. Only caryopses on the first to third primary rachis branches from the top of the panicle were sampled, and were immediately frozen in liquid nitrogen and stored at –80°C until use. Some of the samples were lyophilized, weighed and used for determining sugar contents.

cDNA cloning

To isolate cDNA fragments of rice cell wall invertase, we designed a pair of degenerate PCR primers, CWIU4: TTCTACCARTACAAYCCMAA and CWIL3: AARTCNGGRCACTCCCACAT according to the conserved domain of the amino acid sequences of known cell wall invertases, FYQYNPK and MWECPDF, respectively (see Fig. 1A). Total RNA from rice panicle at the early to mid-ripening stage was subjected to reverse transcription with the oligo-dT13 primer by Super Script II reverse transcriptase (Life Technologies, Rockville, MD, U.S.A.), and the resultant first strand cDNA was used as a template for PCR with the primers described above. An amplified band of about 0.5 kbp was subcloned and sequenced. Since the deduced amino acid sequence of this cDNA fragment showed 80 and 72% identities with the corresponding regions of maize Incw1 and Incw2, respectively, we identified this as a partial sequence of putative rice CWI. Then, this cDNA fragment was used for screening a cDNA library constructed from the same rice panicle RNA as used in RT-PCR with a ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA, U.S.A.) using ECL Direct Nucleic Acid Labeling and Detection System (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). From approximately 5×10^5 independent recombinant phages, a cDNA of 1914 bp was finally selected and sequenced. Although this cDNA clone, pCWI1-2, starting three bases downstream from the 5′ end of the PCR fragment was used as a probe, the deduced amino acid sequence shared again 78 and 73.5% identity with the corresponding regions of maize Incw1 and Incw2, respectively. To obtain the sequence of the 5′ region of pCWI1-2, we performed 5′RACE-PCR using a commercial oligo-capped cDNA from rice leaves (Cap Site cDNA, Nippon Gene, Tokyo) as a template and a primer set 2RC: GTACGCCACAGCGTATGAGC (a primer for the adapter) and INCAP1: CAGAGCCAGACCAACAACCGTA. The resultant DNA band of about 400 bp was subcloned and named pINCAP1. The nucleotide sequence of 85 bp of the 3′ end of pINCAP1 completely matched the corresponding 5′ region of pCWI1-2, indicating that pINCAP1 and pCWI1-2 are derived from the same gene. To confirm this, we designed PCR primers, CINL1: GCCGGCCTGTCTAGTACAAA and CINR1: TATATCGCAGATGGCGAGTG from the nucleotide sequences of the 5′- and 3′- non-coding region of pINCAP1 and pCWI1-2, respectively, and used them for RT-PCR with total RNA from rice panicle as a template. The resultant DNA band of about 1.6 kbp was sequenced, and as expected, the nucleotide sequence of this cDNA fragment was identical to the corresponding region of both pINCAP1 and pCWI1-2. We then designated the assembled sequence *OsCIN1* as a cDNA for a putative CWI gene.

All sequences were analyzed using Genetyx-Win computer software (Software Development Co., Tokyo), and the N-terminal signal sequence was analyzed using SignalP and P-sort (http://www.expasy.ch).

Northern blot analysis

RNA was prepared from various tissues of rice plants using the method of Chang et al. (1993) with both polyvinylpyrrolidone and spermidine omitted from the extraction buffer. Total RNA samples (5 μg per lane) were separated on 1.2% agarose/formaldehyde gels, transferred to nylon membrane and hybridized with digoxigenin (DIG)-labeled riboprobes following the instruction of DIG Northern Starter Kit (Roche). A 382 bp PCR fragment of 3′ non-coding region of pCIN1-2 was prepared using a primer set of CWI15: AGAAGCCGCTTATGAATGG and CWI13: CATGATGAAATAATCAGCATCT. After a T7 RNA polymerase promoter was added using a Lig'n scribe promoter addition kit (Ambion, Austin, TX, U.S.A.), the PCR fragment was used as a template for in vitro transcription, incorporating DIG-UTP. The probes for the 3′ non-coding sequence of putative rice CWI, *INV1* (accession No. AF155121), and rice sucrose transporter, *OsSUT1* (Hirose et al. 1997), generated in the same way were also used.

In situ hybridization

Plant materials were fixed in 4% (w/v) paraformaldehyde and 0.25% (v/v) glutarardehyde in 100 mM sodium phosphate buffer (pH 7.4) overnight at 4°C, dehydrated through an ethanol series and *t*-butanol series, and finally embedded in Paraplast Plus (Sherwood Medical, St. Louis, MO, U.S.A.). Seven μm thick sections were used for in situ hybridization following the method of Kouchi and Hata (1993) with slight modification. The sections were deparaffinized and treated

with proteinase K (5 μg ml^{-1}) in 100 mM Tris-HCl, 50 mM EDTA at 37°C for 30 min, followed by post-fixation with 4% (w/v) paraformaldehyde in 10 mM phosphate buffer pH 7.2. Subsequently, they were treated with 0.5% (v/v) acetic anhydride in 100 mM triethanolamine, pH 8.0 for 10 min, washed in 2× SSPE, and then in distilled water. The sections were then hybridized with the DIG-labeled RNA probes described above at 50°C overnight in a hybridization buffer containing 50% (v/v) formamide, 0.3 M NaCl, 10% (v/v) dextran sulfate, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM DTT and 1 mg ml^{-1} yeast tRNA. After hybridization, the sections were washed with 4× SSC at 50°C, treated with RNase A (20 μg ml^{-1}) in 0.5 M NaCl, 10 mM Tris-HCl, 5 mM EDTA, pH 7.5 at 37°C for 30 min and washed again in 0.5× SSC at room temperature. The hybridized signal was immunologically detected using an anti-DIG-alkaline phosphatase conjugate (Roche) with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indol phosphate toluidinium salt as the substrate following incubation in the blocking solution containing 50% (v/v) normal rabbit serum, 50 mM Tris-HCl, pH 7.5, 75 mM NaCl and 0.5% (v/v) Tween-20.

Assay for cell wall invertase activity

The caryopses with the lemmas and paleae removed were ground with mortar and pestle in an extraction buffer containing 100 mM HEPES-NaOH (pH 7.5), 8 mM $MgCl_2$, 2 mM K_2PO_4, 2 mM EDTA, 12.5% (v/v) glycerol and 50 mM 2-mercaptoethanol. The homogenate was centrifuged at 14,000×*g* for 10 min and the pellet was washed twice in the extraction buffer. After the second wash the pellet was resuspended in the extraction buffer and used for invertase activity assay. An aliquot of the cell wall suspension was added to the assay mixture containing 200 mM Na-acetate (pH 4.5) and 20 mM sucrose and incubated for 20 min at 37°C. The reaction was stopped by boiling for 3 min and resultant glucose was determined using F-kit (Roche).

Sugar content determination

Soluble sugars were extracted from the caryopses in boiling 80% (v/v) ethanol and the concentrations determined enzymatically using F-kit (Roche).

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