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GENETICS AND GENOMICS

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Molecular cloning and expression analysis of the cell-wall invertase gene family in rice (*Oryza sativa* L.)

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Abstract Cell-wall invertase (CIN) catalyzes the hydrolysis of sucrose into glucose and fructose for the supply of carbohydrates to sink organs via an apoplastic pathway. To study the *CIN* genes in rice (*Oryza sativa* L.), we isolated cDNA clones showing amino acid similarity to the plant cell wall invertase proteins from a search of rice sequence databases. Profile analyses revealed that the cloned genes are expressed in unique patterns in various organs. For example, transcripts of *OsCIN1, OsCIN2, OsCIN4,* and *OsCIN7* were detected in immature seeds whereas *OsCIN3* gene expression was flower-specific. Further transcript analysis of these genes expressed in developing seeds indicated that *OsCIN1*, *OsCIN2*, and *OsCIN7* might play an important role involving sucrose partitioning to the embryo and endosperm. Sucrose, a substrate of CINs, induced the accumulation of *OsCIN1* transcripts in excised leaves and *OsCIN2* in immature seeds, while the level of *OsCIN5* was significantly down-regulated in excised leaves treated with sucrose. Infecting the tissues with rice blast (*Magnaporthe grisea*) as a biotic stressor increased the expression of *OsCIN1*, *OsCIN4*, and *OsCIN5*, suggesting that these genes may participate in a switch in metabolism to resist pathogen invasion. These results demonstrate that *OsCIN* genes play diverse roles involving the regulation of metabolism, growth, development, and stress responses.

Keywords Carbon partitioning · Cell-wall invertase · *Magnaporthe grisea* · *Oryza sativa* · Sucrose

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Introduction

In most plants, assimilates, generally sucrose, are transported from the source leaves to the developing sink organs. Uptake in these sinks can occur either directly or through the cleavage of sucrose. Plants contain two types of enzymes capable of this cleavage: (1) sucrose synthase, which catalyzes a readily reversible reaction; and (2) invertase, which catalyzes the irreversible hydrolysis of sucrose to glucose and fructose (Copenald 1990).

The invertases of higher plants are classified according to their solubility, localization, and pH optima, and include three types of enzymes: cytoplasmic, vacuolar, and cell-wall. Vacuolar and cell-wall invertases (CINs) have similar enzymatic properties and two conserved sequence motifs, i.e. the β-fructosidase motif (NDPNG/A) and the cysteine catalytic site (Kim et al. 2000). These two classes of invertases can be reliably distinguished by a single amino acid difference in their cysteine catalytic sites (MWECP/V), with CINs having a proline residue in the sequence motif, and vacuolar invertases possessing a valine residue (Goetz and Roitsch 1999; Tymowska-Lalanne and Kreis 1998).

CINs are most active in the apoplast of sink organs, and can be involved in sucrose partitioning, control of cell differentiation and plant development, and responses

to wounding and pathogen infection (Roitsch et al. 2003; Sturm 1999; Sturm and Tang 1999; Tymowska-Lalanne and Kreis 1998). This class of invertase has been isolated from several plant species, e.g. carrot (Lorentz et al. 1995; Unger et al. 1994), *Arabidopsis* (Tymowska-Lalanne and Kreis 1998), maize (Xu et al. 1996), potato (Maddison et al. 1999), tobacco (Goetz et al. 2001), tomato (Godt and Roitsch 1997), and Japanese pear (Hashizume et al. 2003). In the *Arabidopsis* genome, six putative *CIN* genes have been identified (Sherson et al. 2003), while four, *Incw1–Incw4,* have been reported in maize (Kim et al. 2000; Shanker et al. 1995; Taliercio et al. 1999). For example, the maize *miniature-1* seed mutant, associated with aberrant pedicel and endosperm development, has decreased invertase activity in these organs and reduced expression of *Incw2* (Cheng et al. 1996; Miller and Chourey 1992). Finally, two barley CINs, *HvCWINV1* and *HvCWINV2*, have been isolated and shown to play important roles in controlling sugar ratios in maternal and filial tissues during the early development of caryopses (Weschke et al. 2003).

OsCIN1, a rice CIN gene, is active in developing seeds (Hirose et al. 2002). During the grain-filling stage in the caryopsis, its expression is detectable only at 1–4 days after flowering (DAF). In situ localization of 1-DAF caryopses has shown that *OsCIN1* mRNA is expressed mainly in the maternal tissues. These results suggest that this gene has a critical role during the pre-storage phase, being involved in the proliferation of endosperm cells and longitudinal growth of immature seeds, rather than during the starch-filling phase. Therefore, it is most probable that the function of another invertase is required for the remaining activity after 5 DAF, when the caryopses are developing.

CIN activity can be increased by such phytohormones as auxins (Morris and Arthur 1984), gibberellic acids (Wu et al. 1993), cytokinins (Ehneß and Roitsch 1997), brassinosteroids (Goetz et al. 2000), and abscisic acid (Richings et al. 2000). In contrast, ethylene represses the expression of *CIN* genes (Linden et al. 1996). Activity of these genes is regulated by sugars (Godt and Roitsch 1997; Krausgrill et al. 1996; Tymowska-Lalanne and Kreis 1998) or by several stress-related factors, including pathogens (Hall and Williams 2000; Herbers et al. 2000; Sturm and Chrispeels 1990), wounding (Matsushita and Uritani 1974), osmotic stress (Wang et al. 2000), or cold(Balk and de Boer 1999).

In the present study, we sought to identify CIN genes from the recently published sequence of the rice genome. To do so, we isolated all their cDNA clones and grouped them with known genes to investigate an evolutionary relationship. Their genomic structures were then determined by comparing the cloned cDNAs with published genomic sequences. To understand the possible roles of *CIN* genes, we also investigated temporal and spatial expression of these cloned genes in various rice organs, including the leaves, roots, flowers, and developing seeds. Finally, we examined the responses of these rice CIN (*OsCIN*) genes to increased levels of sucrose as well as to pathogen attack.

Materials and methods

Plant materials

Greenhouse-grown japonica rice (*Oryza sativa* L.) variety Jinmi was used in all experiments, except for the rice blast tests, in which we infected 3-week-old leaves of variety RIL260. Immature seeds were harvested at different developing stages and flowers were also gathered prior to heading. The flag leaves of mature plants were collected to represent a source organ while roots were taken at the four-leaf seedling stage. All samples were frozen in liquid nitrogen and kept at −80°C until needed.

cDNA cloning

Full-length cDNAs of the *OsCIN* genes were isolated by RT–PCR analysis, using gene-specific primers encompassing the translation start codon and 3′-untranslated regions. Primer pairs included: for *OsCIN1*, 5′-TCTAGTACAAAACAATGGGGACTC-3′ and 5′-CGGAAAACCTCTTTATTATCTGTA-3′; for *OsCIN2*, 5′-CTCTCCTCTTCTCGCTCTCACTTC-3′ and 5′-TGACTGAAACCATTTTACACAAGG-3′; for *OsCIN3*, 5′-AGCGAGGTAAGTAGTGTGTTAGTG-3′ and 5′-GTGTTAAAACTGGACTTATTTTGG-3′; for *OsCIN4*, 5′-CAAGTTAACAAAGTCTTTCACACA-3′ and 5′-GATTTCCATCTCCTTTCTACAAGT-3′; for *OsCIN5*, 5′-AAAGTCTCGTCCTGTTCATCTTCT-3′ and 5′-TTTTATCCTGGGAATAAGGCAATA-3′; for *OsCIN6*, 5′-AATTTGCAAACTCTACTGTTTTGT-3′ and 5′-TGCTAAAAAGTCAATAGTGTCAAA-3′; and for *OsCIN7*, 5′-ACCAAACAGGCGTTTTCTTCAGAG-3′ and 5′-GCAAAACATATAAAGCACGTGACG-3′. The cDNAs synthesized with mRNA isolated from the leaf, root, flower, and immature seed were used in the PCR reactions.

Sequence alignment and phylogenetic tree construction

Deduced amino acid sequences of the rice CINs were aligned with reported genes from other species, using the CLUSTAL W program (Thompson et al. 1994). Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 2.1 (Kumar et al. 2001). The accession numbers of the sequences used to construct the phylogenetic tree are: *AtcwINV1–AtcwINV6* (NM_112232, NM_115120, NM_104385, NM_129177, NM_112231, NM_121230, respectively) from *Arabidopsis; DcInv1* (M58362) and *DcInv2* (X78424) from carrot; *ZmIncw1–ZmIncw4* (U17695, AF050631, AF043346, AF043347, respectively) from maize; *StInv1* (Z22645) from potato; *Psbfruct1* (X85327) from pea; *Ntbfruct1* (X81834) and *NtNin88* (AF376773) from tobacco; and

HvCWINV1 (AJ534447) and *HvCWINV2* (AF155121) from barley.

Analysis of genomic structure and chromosomal location

The genomic structure of each *OsCIN* gene was determined by aligning the cDNA sequences and genomic sequences of BAC/PAC clones obtained from the NCBI database. The accession numbers of those clones used to identify structure and chromosomal location are: for *OsCIN1*, AP004156; for *OsCIN2* and *OsCIN3*, AL662945; for *OsCIN4*, AP004365; for *OsCIN5* and *OsCIN6*, AL606646; and for *OsCIN7* and *OsCIN8*, AP005738. The *OsCIN* genes were located on a high-density genetic map (Wu et al. 2002), using the Integrated Rice Genome Explorer (INE) database (http://rgp.dna.affrc.go.jp/giot/INE.html), and the fingerprinted contig (FPC) map (http://www.genome.arizona.edu/fpc/rice)

RNA isolation and semiquantitative RT–PCR analysis

Total RNA was prepared from various organs using Trizol reagent (Invitrogen, http://www.invitrogen.com). The isolated RNA was reverse-transcribed with an oligo-dT primer and the First Strand cDNA synthesis kit for RT–PCR (Roche, http://www.roche-applied-science.com). First-strand cDNA was used in PCR reactions with gene-specific primers and control primers, including *OsGBSSII* (Dian et al. 2003), *Act1* (McElroy et al. 1990), 18S rRNA (Kim et al. 2003), and the pathogenesis-related probenazole-inducible (*PBZ1*) genes (Midoh and Iwata 1996). Gene-specific primers were designed in the region encompassing at least one intron per gene to exclude any influence by genomic DNA contamination. These primers were: for *OsCIN1*, 5′-CGACCCTACCAA GTCTTCTCTTAG-3′ and 5′-CCCATTGTTGAAGACG TAAAGATG-3′; for *OsCIN2*, 5′-GAAGATATCTCTG AGGAGCCTGAT-3′ and 5′-TAGGCTCCATTCATCAT GACC-3′); for *OsCIN3*, 5′-CATCAAGAAGGGCAACT ACTTC-3′ and 5′-GTTGTTGAAGACGAAGAGGTG-3′; for *OsCIN4*, 5′-GAGATCAAGGGAACTGATACTTTA-3′ and 5′-CATGTACTTCTGCTCTGATTTGTA-3′; for *OsCIN5*, 5′-ATGGAAAGTACAAGGTCTTCATGT-3′ and 5′-ATTGTTGAACACGTACAGGTGACT-3′; for *OsCIN6*, 5′- ATTCGTGGACATAGACATAGAGAA-3′ and 5′-ATACAGGACCACCATACGAAAT-3′; for *OsCIN7*, 5′-AGGAGCAGGTCCAGAACGTC-3′ and 5′-CC GTTGTTGAACACGTACAAAT-3′; for *OsGBSSII*, 5′-T AGGTGTCACTGAATGGCTAGAAC-3′ and 5′-TGGC CCACATCTCTAAGTAACATC-3′; for *Act1*, 5′-GGA ACTGGTATGGTCAAGGC-3′ and 5′-AGTCTCATGG ATACCCGCAG-3′; for 18S rRNA, 5′-ATGATAACTCGA CGGATCGC-3′ and 5′-CTTGGATGTGGTAGCCGTTT-3′; and for *PBZ1*, 5′-ACCATCTACACCATGAAGCT TAAC-3′ and 5′-GTATTCCTCTTCATCTTAGGCGTA-3′.

In the PCR analysis of seed coat and endosperm, each total cDNA isolated by mass in vivo excision from seed coat- and endosperm-specific libraries was used. These cDNA libraries were made with mRNA from the seed coats and endosperm of 6- to 10-DAF rice (Jun et al. 2004; S.-H. Jun et al., unpublished data). For PCR, the amplification program consisted of an initial 94°C for 5 min followed by 28–35 cycles of 94°C, for 1 min; 56°C, for 1 min; 72°C, for 1 min, and a final extension at 72°C for 5 min. Experiments were repeated at least 3 times to obtain similar results.

Sucrose treatment

Seedlings at the four-leaf stage were maintained for 48 h under dark conditions to deplete their endogenous sugars. Leaves were then excised in 1-cm portions and treated in the dark at 28°C with MS media containing either 175 mM sucrose or mannitol (Dian et al. 2003).

Rice blast infection

We inoculated 3-week-old rice leaves with *Magnaporthe* grisea avirulent isolate PO6-6, according to the method of Jeon et al. (2003). These infected leaves were then collected periodically for RT–PCR analysis.

Results

cDNA cloning of rice cell wall invertase genes

To identify all the CIN genes in the rice genome, we carried out systematic BLAST searches of its published sequence (Feng et al. 2002; Goff et al. 2002; Sasaki et al. 2002; Yu et al. 2002), using the nucleotide and amino acid sequences of the rice *OsCIN1* gene (Hirose et al. 2002), maize *Incw2* (Cheng et al. 1996; Miller and Chourey 1992), and *Arabidopsis* CINs (Sherson et al. 2003). Eight putative CIN genes in rice were identified as *OsCIN2*–*OsCIN8* to follow the previously isolated rice CIN gene *OsCIN1*. Full-length cDNAs of seven of these *OsCIN* genes were isolated by RT–PCR analysis using gene-specific primers that encompassed the translation start codon and 3′-untranslated regions. Endogenous transcript for the *OsCIN8* gene in the various samples, including the sucrose-treated and the rice blast-infected tissues, was not detectable, indicating that the gene is inactive in the tissues examined.

Based on our CLUSTAL W alignment of the deduced amino acid sequences, all the identified *OsCIN* genes showed two conserved sequence domains—the β-fructosidase motif (NDPNG/A) and the cysteine catalytic site (Kim et al. 2000)—and all possessed a proline residue in the latter (Fig. 1). Therefore, we grouped these *OsCIN* genes in the classification of CINs.

```
OsCIN1   -------MGTRLLALAPWLLLLLLQLAG----------ASHVVHRSLEAEQAPS---SVPASIVSPLLRTGYHFQPPMNWINDPNGPLYY
OsCIN2   ----MGVLGSRVAWAWLVQLLLLQQLAG----------ASHVVYDDLELQAAATTADGVPPSIVDSELRTGYHFQPPKNWINDPNAPMYY
OsCIN3   ----MATARARAALVFVALLQMAAVVVVR---------ASHVVYPELQSLEAKH---------VDGKLRTGYHFQPPKHWINDPNGPMYY
OsCIN4   MVMAPIPQPWHQWPFLILFFLVLFSCESNLPCRNGVEATQRVFLYPQSPKVSSI---------VSKGYRTGYHFQPPKNWINDPNGPMYY
OsCIN5   -----------------------------------------MCPVNGKL---------QLHDGRTAYHFQPAKFWQNDPNGPLYH
OsCIN6   --MALAGLPLSVFAIAVHFCLVFSSSSS----------------PPVCPANG-------------HRDRTAYHFQPAKNWQNDPNGPVYY
OsCIN7   ----MARLGLAVCAASFHLFLLLASTSS----------------LRRAPTEADT---------ANHARRTAYHFQPAKNWQNDPNGPMYH
OsCIN8   ----MARLGLAVCAVSFHLCLLLSSTSS----------------LRLNPTTEDT---------ANHGRRTAYHFQPAKNWQNDPNGPLYH
                                                                                **.*****.  * ****.*:*:
                                                                                β-Fructosidase motif

OsCIN1   KGWYHLFYQYNPKGAVW--GNIVWAHSVSQDLINWIALEPAIKPDIPSDQYGCWSGSATILPDGTPAILYTGIDRPNINYQVQNIAFPKN
OsCIN2   KGWYHLFYQYNPKGAVW--GNIVWAHSVSRDLINWVALKPAIEPSIRADKYGCWSGSATMMADGTPVIMYTGVNRPDVNYQVQNVALPRN
OsCIN3   KGLYHLFYQYNPKGAVW--GNIEWAHSVSTDLIDWTALEPGIYPSKTFDEKGCWSGSATVLPSGVPVIMYTGID-PDE-RQVQNVAYPVN
OsCIN4   NGIYHEFYQYNPNGSVW--GNIVWGHSVSTDLINWIRLEPAIEGNTPSDINGCWTGSATILTGDQPVIIYTGADTEKR--QVQNIVLPKN
OsCIN5   NGLYHFFYQYNPHGPLWDTGKLSWGHSVSGDLVNWAFLGTAIDPTDPFDVNGCWSGSATVLLGGRPAFLYTGRDAGGV--QVQNVSFAKN
OsCIN6   NGMYHLFYQYNPHGALWDVGNLSWGHSVSGDLVNWAALDNALDPTAPFDANGCASGSVTILPDGVPVVMYSGIDARRR--QVQNVAFPKN
OsCIN7   NGMYHLFYQYNPHSALWDIGNLSWGHSVSGDLLNWAALDTALDPTSPFDANGCWSGSATILPGALPAILYTGIDASKE--QVQNVAFAKN
OsCIN8   NGMYHLFYKYNPHSALWDIGNLSWGHFVSGDLLNWAALDTALDPTSPFDANGCWSGSATILPGGLPAILYTGIDAGKE--QVQNVAFAKN
          :* ** **:***:..:*  *:: *.* ** **::*  *  .:      *  ** :**.*:: .  *..:*:* :      ****:  . *

OsCIN1   ASDPLLREWVKPAYNPVAT-PEPGMNATQFRDPTTAWY-ADGHWRMLVGGLKGARLGLAYLYRSRDFKTWVRAKHPLHSALTG-MWECPD
OsCIN2   GSDPLLREWVKPGHNPVIV-PEGGINATQFRDPTTAWRGADGHWRLLVGSLAGQSRGVAYVYRSRDFRRWTRAAQPLHSAPTG-MWECPD
OsCIN3   LSDPYLREWYKPDYNPIIN-PDGGINASAFRDPTTAWYGPDGHWRLLVGSKVN-MKGLAVLYRSRDFKKWVKAHHPLHSAHTG-MWECPD
OsCIN4   RSDPYLREWTKPKNNPLIEPVGPGLNSNQFRDPTTGWIGPDGLWRIAVGAELN-GYSAALLYKSKDFMQWTRVDHPLYSSNASNMWECPD
OsCIN5   PLDPLLREWEKPSCNPIIA-FPADVINNNFRDPTTAWLGRDGLWRMVVAAEVA-GAGSALVYRSADFLRWERNAAPMHSSAAVPVLECPD
OsCIN6   PRDPLLREWTKPGYNPVIP-VPADVSPDNFRDPTTAWLGSDGLWRFAISAVAD-GVGATLVYRSADFLRWERNAAPLHASRDAVMAECPD
OsCIN7   PSDPLLREWEKPAYNPVIA-LPADVPGDKFRDPSTAWLGRDGLWRIAVSAEVD-GVASTLVYRSKDFVRWERNAAPLHASRAAGMVECPD
OsCIN8   PSDPLLREWEKPAYNPVIA-LPPDVPGDNFRDPSTAWLGRDGLWRIAVSAEVD-GVASTLVYRSEDFVRWERNAAPLHASRAAGMVECPD
          ** **** **  **:      .:    ****:*.*   ** **: :..     . : :*:* **  * :   *::::  : ****
                                                                                Cys catalytic site

OsCIN1   FFPLQAPG----LQAGLDTS------V-PSS--KYVLKNSLDLTRYDYYTVGIYNKVTERYV--PD---NPAGDYHR-LRYDYGNFYASK
OsCIN2   FYPVTADG----RREGVDTSSAVVDAA-ASARVKYVLKNSLDLRRYDYYTVGTYDRKAERYV--PD---DPAGDEHH-IRYDYGNFYASK
OsCIN3   FFPVAVAGGSRHYRRGVDTAELH-DAA-VAEEVKYVLKVSLDLTRYEYYTVGWYDHATDRYV--PD---AAFPDNDYGLRYDYGDFYASK
OsCIN4   FFAVLPGK-----NNGLDLSAAIPN------GAKHVLKMSLDS--CDKYMIGVYDLKHDMFV--PD---TVLDDRRLWLRIDYGNYYASK
OsCIN5   FFPVAEHG-----IDGLDTSANGGG-----TGVKHVLKLSEFDTHQDFYMVGRNRRVQWLWVNEYD---SKADDVA------------K
OsCIN6   LFPVAEHG-----EDGLDLDASAIG--GAGAGVRHVLKVSMPDTLEDYYMVGRYDDADDTFTVPPEDLEAHGDDYRRWRRIDHGHLYASK
OsCIN7   LFPVAERG-----EDGLDTSANGAG------GVRHVLKLSVMDTLQDYYMVGTYDDAADAFS-PAE--PERGDDCRSWRRLDYGHVYASK
OsCIN8   MFPVAENG-----EDGLDTSTNGAG------GVRHVLKLSVMDTLQDYYMVGTYDDAADAFS-PAE--PERGDDCRRWRRLDYGH-----
          ::.:          *:*                ::*** *       : * :*      :    :       *

OsCIN1   TFFDPVKHRRILLGWANESDSVTYDKAKGWAGIHAIPRKVWLDPSGKQLLQWPIEELETLRGKS--VSVFDKVVKPGEHFQVTGLGTYQA
OsCIN2   TFYDPAKRRRILWGWANESDTAADDVAKGWAGIQAIPRKVWLDPSGKQLLQWPIEEVERLRGKWP-VILKDRVVKPGEHVEVTGLQTAQA
OsCIN3   SFYDPAKRRRIVWGWANESDTVPDDRRKGWAGIQAIPRKLWLSADGKQLVQWPVEELKALRAKH--VNVTDKVIKKGNYFEVTGFKSVQS
OsCIN4   SFFDSKKGRRIIWGWTNETDSTSDDVAKGWAGIHAIPRTIWLDGDGKRLLQWPIEEVESLRRNE--VSHQGLELKKGDLFEIKGTDTLQA
OsCIN5   ------------------G---------WAGVQAFPRKVWLDGDGKQLLQWPVDEIETLRTKR--VGLQGTEVKAGGLHEIVGVASSQA
OsCIN6   TFYDAGKKRRVLWAWVNESDSEADDVTKGWSGLQSFPRAVWLDEGGRQLVQWPVEEIETLRRKR-GVLLGGNEVEAGGLREIGGIAGSQA
OsCIN7   SFFDVRKNRRVLWAWANESDSQADDVARGWSGVQTFPRKMWLAKDGKQLLQWPIEEIKTLRRKR-AGLWQGTRLGAGAVQEIVGVASSQA
OsCIN8   ----------------------------------TFPRKMWIAKDGKQLLQWPIEEIETLRRKRAAGLRRGTRLGAGAVQEIVGVASSQA
                                           ::** :*:  .*::*:***::*:: ** :       .  :  *   :: *   *:
          Conserved glycosylation motif

OsCIN1   DVEVSLEVSGLEKAEA-LDPAFGDDAERLCGAKG-ADVRGGV-VFGLWVLASAGLEEKTAVFFRVFKPAGHG---AKPVVLMCTDPTKSS
OsCIN2   DVEVSFEVGSLEAAER-LDPAMAYDAQRLCSARG-ADARGGVGPFGLWVLASAGLEEKTAVFFRVFRPAARGGGAGKPVVLMCTDPTKSS
OsCIN3   DVDMAFAIKDLSKAEE-FDPAWRTDAEALCKKLG-SDVDGGVGPFGLWALASGDLKERTAVFFRVFKANDSS-----HVVLMCNDPTRSS
OsCIN4   DVEIDFELTSIDAADP-FDPSWLLDTEKHCREADAS-VHGGLGPFGLVVLASDNMDEHTTVHFRVYKSEQKY------MVLLCSDLRRSS
OsCIN5   DVEVVFEIPNLEDEAESFDPD-WLDPHKLCKDKGAASAHGGVGPFGLIVMASGDLQEQTAVFFRVFKHHGKY------KVFMCTDLTRSS
OsCIN6   DVEVAFEIASLAGADR-LEPDHLRDPDALCGENGAA-VHGGIGPFGLLVMASGDLRERTAVFFRVFRLSHGY------TVLMCTDLTRST
OsCIN7   DVEVVFKIPSLEEAERVDDPNRLLDPQKLCGEKGAA-VRGGVGPFGLLVMASGDLHEHTAVFFRVFRHHDKY------KLLMCTDLTKSS
OsCIN8   DVEV--------------------------------------------------------------------------------------
          **::

OsCIN1   LSPDLYKPTFAGFVDTDISSGK-ISLRSLIDRSVVESFGAGGKTCILSRVYPSMAIGDKAHLYVFNNGEADIKISHLKAWEMKKP-LMNG
OsCIN2   RNPNMYQPTFAGFVDTDITNGK-ISLRSLIDRSVVESFGAGGKACILSRVYPSLAIGKNARLYVFNNGKAEIKVSQLTAWEMKKPVMMNG
OsCIN3   YESKIYRPTFAGFVDVDIAKNKQIALRTLIDHSVVESFGARGKTCILTRVYPRKAVGDDAHLFVFNNGESDVKVTNLDAWEMKTP-KMNA
OsCIN4   LRPGLYTPAYGGFFEYDLEKEKKISLRTLIDRSAVESFGGGGRACIMARVYPAAVVDGATHMYAFNNGSSTVKVSQLKAWSMTRAQVN--
OsCIN5   TKADVYKDAYGGFVDVDIQKDKSISLRTLIDHSMIESFGGGGRACITTRVYPEHAATSSSHLYVFNNGSGTVNVSKLEAWEMATATVN--
OsCIN6   SRAGVYKPSHGGFVDIDIEKDRAISLRTLIDHSIVESFGGGGRTCMTARVYPEHVATGSSHLYVFNNASDAVKVSKLEAWELATASVN--
OsCIN7   TRAGVYKPAYGGFVDMDIDDHKTISLRTLIDHSVVESFGGGGRACITARVYPEHVATSSSHLYVFNNGSDAVKVAKLEAWDLATATVNVV
OsCIN8   -----------------------------IDHSVVESFGGGGRACITARVYPEHVATSSSHLYVFNNGSDDVKVAKLEAWDLATATVNVV
                                       **:* :****. *::*: :****  .    ::::.***..  :::::* **.:  .

OsCIN1   A-------------------
OsCIN2   A-------------------
OsCIN3   EE------------------
OsCIN4   ----------------VRKG-
OsCIN5   ---S------ADALDAITRS-
OsCIN6   AGDD-GLISYGGPVCAAQVQ-
OsCIN7   VGDHHGLVAPALELEPTRTTQ
OsCIN8   VGDHHGLAAPALELEPTRTTQ
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Fig. 1 Comparison of deduced amino acid sequences for rice cell-wall invertases (*CIN*s). Conserved β-fructosidase motifs, cysteine catalytic sites, and conserved glycosylation sites are shown in *shaded regions* and are *underlined*. *Bold letters* indicate the proline residue of the cysteine catalytic site. Identical and conserved residues are marked with *asterisks* and *dots/colons*, respectively. Accession numbers of *OsCIN* cDNAs and one gene are: for *OsCIN1*, AY578158; for *OsCIN2*, AY578159; for *OsCIN3*, AY578160; for *OsCIN4*, AY578161; for *OsCIN5*, AY578162; for *OsCIN6*, AY578163; for *OsCIN7*, AY578164; and for *OsCIN8*, AY578165

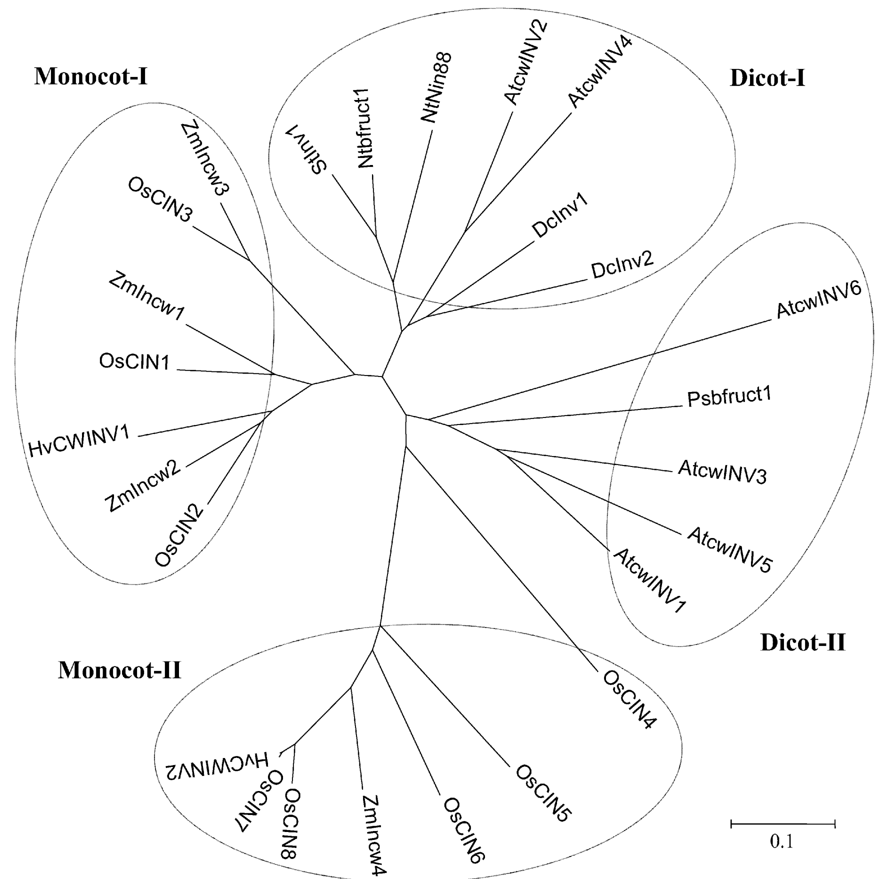

Fig. 2 Phylogenetic tree of CIN genes constructed using the neighbour-joining method. *Scale bar* corresponds to a distance of ten changes per 100 amino acid positions. Accession numbers are described in Materials and methods

Phylogenetic and molecular evolutionary analysis

To establish the phylogenetic relationship of these *OsCIN* genes with CIN genes from different plant species, we constructed an unrooted phylogenetic tree using the neighbour-joining method (Fig. 2). This phylogenetic tree displayed four major groups: monocot-I, monocot-II, dicot-I and dicot-II. The *OsCIN1–OsCIN3* genes belonged to monocot group I along with maize *ZmIncw1–ZmIncw3* and barley *HvCWINV1*. The group IV genes, *OsCIN4–OsCIN7* and *OsCIN8*, could be classified with other monocot-II genes, such as maize *ZmIncw4* and barley *HvCWINV2*.

Genomic structure and chromosomal location of *OsCIN* genes

The inferred gene structure (Fig. 3) of the eight *OsCIN* genes was determined by aligning the cDNA sequences and genomic sequences of the BAC/PAC clones obtained from the NCBI database. Interestingly, all the second exons of our genes were 9-bp long. These exons are the smallest known to function among plant species, and encode three amino acids (DPN) of the β-fructosidase motif (NDPNG/A). Furthermore, they are highly conserved in both monocots and dicots, indicating that this 9-bp exon is a prerequisite for invertase activity.

The genomic sequence and structure of *OsCIN8* was quite similar to those of *OsCIN7*, with both containing a comparatively larger second intron, i.e. 3.1 kb in *OsCIN7* and 6.1 kb in *OsCIN8*. The latter gene has an insertion of a putative transposable element similar to the maize transposon *MuDR* within the second intron, which may cause inactivation of *OsCIN8*. That gene has only four exons for the deletion of a region corresponding to a portion of Exon 3, Intron 3, and Exon 4 of the *OsCIN7* gene.

The chromosomal location of the *OsCIN* genes was based on a high-density genetic map (http://rgp.dna.affrc.go.jp; Wu et al. 2002). We used INE, a database integrating a genetic map, physical map, and sequencing information of the rice genome (Sakata et al. 2000), and the rice FPC map (Chen et al. 2002). Here, *OsCIN4* was mapped to chromosome 1; *OsCIN1* to chromosome 2; *OsCIN2*, *OsCIN3*, *OsCIN5*, and *OsCIN6* to chromosome 4; and *OsCIN7* and *OsCIN8* to chromosome 9 (Fig. 4). *OsCIN2* and *OsCIN3*, *OsCIN5* and *OsCIN6*, and *OsCIN7* and *OsCIN8* were each located in pairs, which suggests that the *OsCIN* genes may have been duplicated during the evolution of the rice CIN genes. This hypothesis is further supported by the fact that each of the two linked genes in a pair retains a relatively higher similarity.

Expression profile analysis of *OsCIN* genes

Spatial expression of the *OsCIN* genes was examined by RT–PCR, using specific primers. Transcripts were detected in particular patterns within the various organ types, i.e.

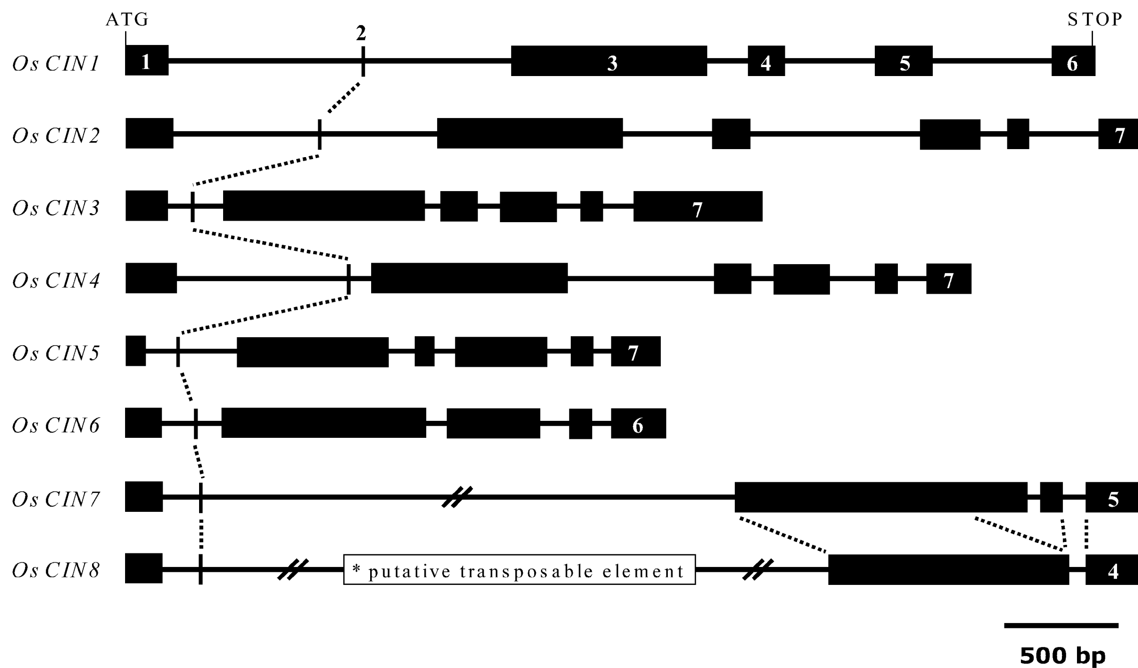

Fig. 3 Genomic structures of members of the *OsCIN* gene family. Exons are indicated as *black rectangles* and introns as *lines*. *Numbers in rectangles* indicate order of exons. Accession numbers of genomic DNAs used to determine the structure of the *OsCIN* gene family are described in Materials and methods

leaf, root, flower, and immature seed (Fig. 5a). *OsCIN1*, *OsCIN4*, and *OsCIN7* were expressed in all tested organs, including a relatively low level measured in the seeds. *OsCIN1* and *OsCIN7* exhibited similar patterns. *OsCIN2* transcript was not detected in the leaves, whereas *OsCIN5* and *OsCIN6* were expressed in the leaves and roots and in the roots and flowers, respectively. Four of the genes, *OsCIN1*, *OsCIN2*, *OsCIN4*, and *OsCIN7*, were expressed in the immature seeds while *OsCIN3* was flower-specific. *OsCIN8* gene was not expressed in any organs.

Because CIN genes are thought to play an important role during seed development in rice and barley (Hirose et al. 2002; Weschke et al. 2003), we further analysed the four genes, *OsCIN1*, *OsCIN2*, *OsCIN4* and *OsCIN7*, that were expressed in immature seeds. Three of these—*OsCIN1*, *OsCIN4*, and *OsCIN7*—were expressed specifically in the seed coats, while transcript of *OsCIN2* was detected in both endosperms and seed coats (Fig. 5b). Our experiment also confirmed that *OsCIN1* mRNA is localized mainly in the maternal tissues of the developing caryopsis (Hirose et al. 2002).

RT–PCR analysis was also performed using a series of seeds collected at different development stages (1–15 DAF) as well as from the ovaries prior to pollination (Fig. 5c). *OsCIN1* was highly expressed in the ovary and at the very early developmental stage of the seed (1–2 DAF). Levels of *OsCIN1* transcript dramatically decreased during 5–8 DAF and were weak thereafter. The expression pattern for *OsCIN1* was similar to that previously reported by Hirose et al. (2002). Likewise, the expression of *OsCIN4* and *OsCIN7* was lowest during 5–8 DAF. *OsCIN7* transcripts levels were high in the starch-filling phase (9–15 DAF), suggesting that this gene may play a role in the sucrose uptake to the endosperm during that phase rather than in the pre-storage phase. In contrast, expression of *OsCIN2* was highest at 1–4 DAF and decreased only slowly afterwards. Notably, this gene maintained a relatively steady level of expression from 5 to 8 DAF, during which time transcripts of *OsCIN1*, *OsCIN4*, and *OsCIN7* were barely detectable. Therefore, we believe that the *OsCIN2* gene has a role distinct from that of the other three genes during endosperm development and longitudinal growth of the seed coat.

Effects of sucrose on expression of *OsCIN* genes

To test the effects of sucrose on *OsCIN* gene expression, excised leaves were treated with 175 mM of either sucrose or mannitol. We included this mannitol treatment in order to eliminate the usual induction caused by osmotic stress. RT–PCR analysis for four genes, *OsCIN1*, *OsCIN4*, *OsCIN5*, and *OsCIN7*, expressed in leaf samples demonstrated that the transcript level of *OsCIN1* was significantly increased by sucrose treatment, while that of OsCIN5 was down-regulated (Fig. 6a). This result indicates that *OsCIN1* may be involved in catalyzing the hydrolysis of sucrose into glucose and fructose for the supply of carbohydrates. Furthermore, the reduced expression of *OsCIN5* because of a higher sucrose concentration suggests that the role of this gene may vary from one of sucrose partitioning.

We also examined the effects of sucrose on gene expression in caryopses at two different stages: 1–2 DAF and 9–10 DAF (Fig. 6b). Our profile analysis showed that sucrose significantly induced expression of *OsCIN2* at both immature stages whereas *OsCIN1*, *OsCIN4*, and *OsCIN7*

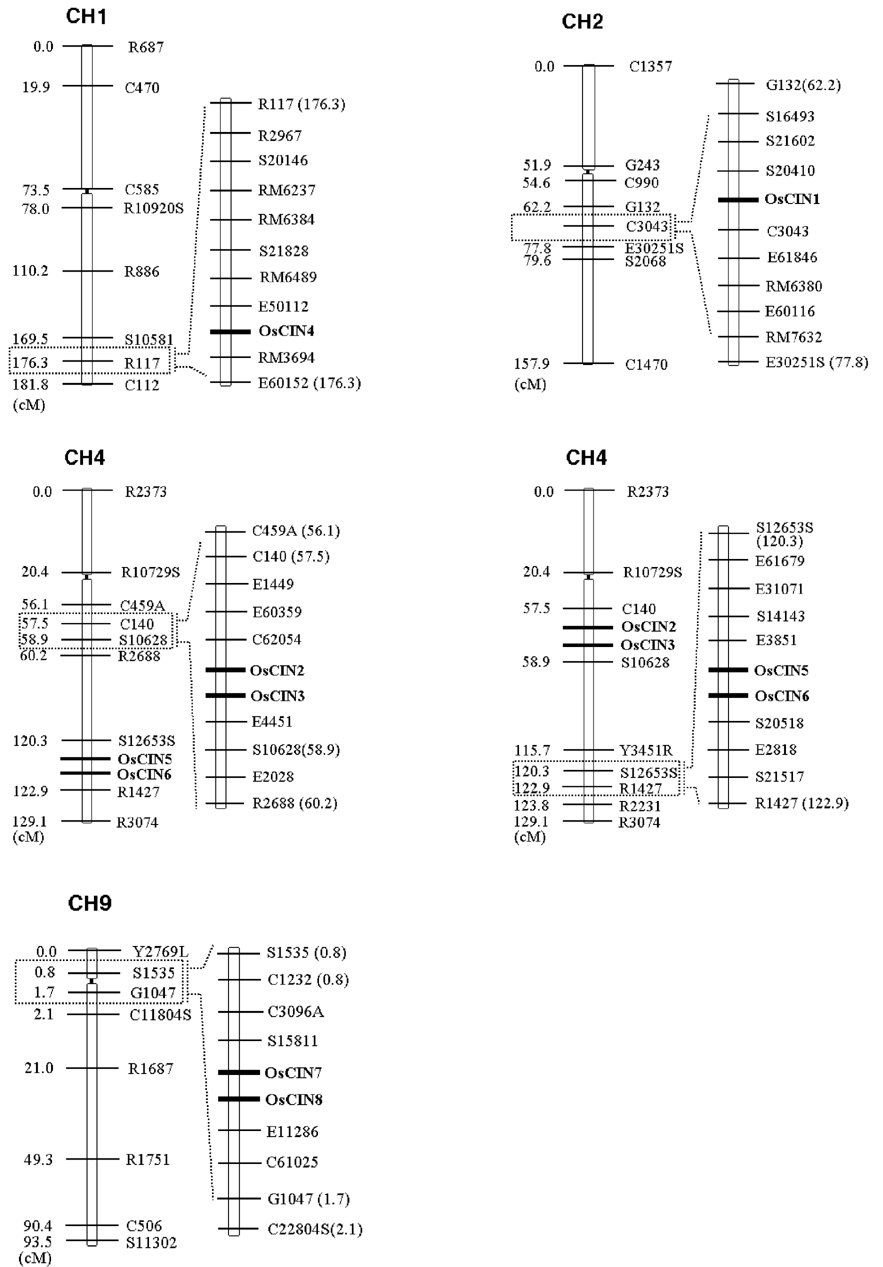

Fig. 4 Chromosomal locations of *OsCIN* genes. Accession numbers of BAC/PAC clones containing these genes are described in Materials and methods

were not affected. Therefore, we postulate that *OsCIN2* plays a predominant role in establishing metabolic sink strength during seed development. The fact that mannitol at the same molarity as used for sucrose had no influence on *OsCIN* activity implies that the change in expression caused by sucrose treatment was not a result of osmotic stress. Dian et al. (2003) have also reported that the expression of *OsGBSSII*, when used as a positive control, can be induced by such sucrose treatment.

Response of *OsCIN* genes to infection by *Magnaporthe grisea*

CIN genes may be directly involved in various plant-pathogen interactions (Hall and Williams 2000; Herbers et al. 2000; Sturm and Chrispeels 1990). In fact, we found considerable EST clones corresponding to *OsCIN1* and *OsCIN4* from cDNA libraries of leaves infected with rice blast (data not shown). Therefore, to determine whether expression in the *OsCIN* gene family is affected by pathogen attack, we examined four genes, *OsCIN1*, *OsCIN4*, *OsCIN5*, and *OsCIN7*, that were expressed in leaf samples (Fig. 7). Here, transcript levels of *OsCIN1* rapidly increased at 4 h after blast infection. In contrast, *OsCIN4* transcripts rose slightly 4 h after infection, peaked at 24 h, and then slowly declined to a low level. While *OsCIN5* expression reached a maximum level at 48 h after infection, that of *OsCIN7* showed no remarkable change after such treatment. Transcript of the pathogen-inducible gene *PBZ1* was significantly increased at 24 h after rice blast infection while the

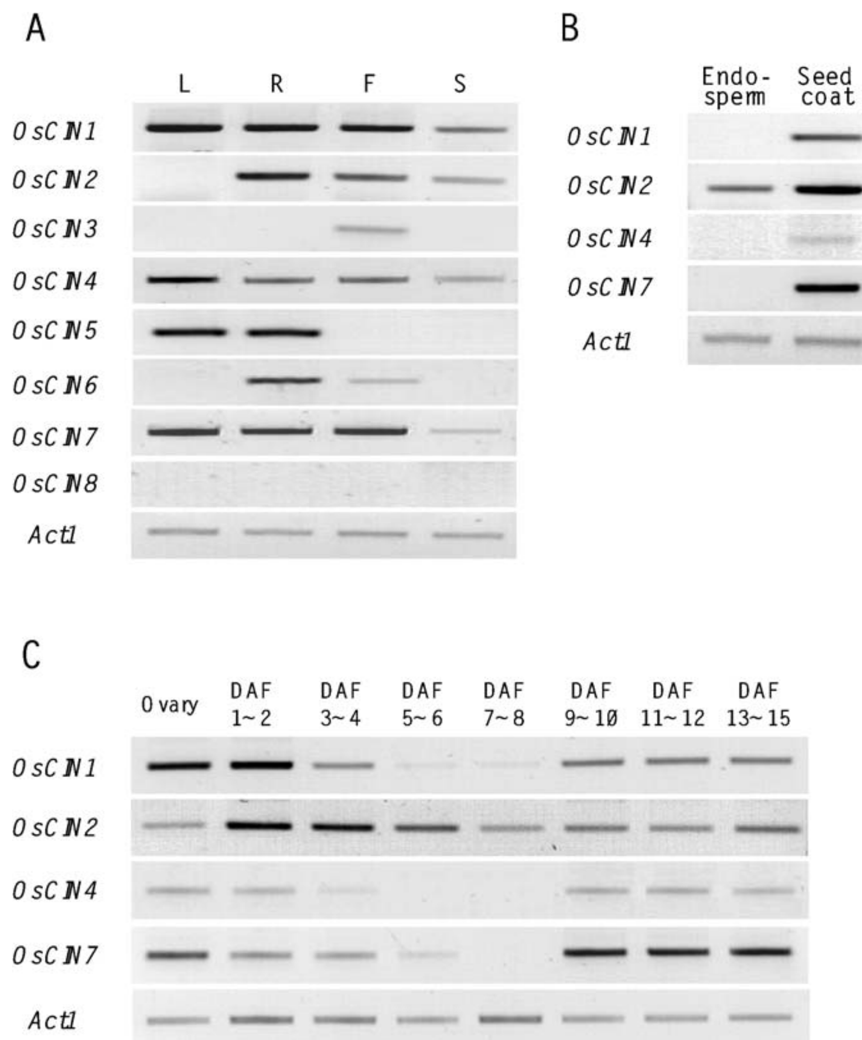

Fig. 5a-c Expression profiles of *OsCIN* genes. For each gene, transcript levels in different tissue samples are comparable but the comparison of transcript levels among genes is not appropriate in the PCR reactions. **a** Spatial expression patterns of *OsCIN* genes in rice tissues, including leaf (*L*), root (*R*), flower (*F*), and immature seeds (*S*) mixed from 1- to 12-days after flowering (*DAF*) caryopses. **b** Expression patterns of *OsCIN* genes in endosperm and seed coat. **c** Changes in gene expression levels of *OsCIN1*, *OsCIN2*, *OsCIN4* and *OsCIN7* in rice seeds during development. Rice seeds were harvested over a time course of 1–15 DAF

Act1 gene, used as a PCR control, did not alter its expression in response to the inoculation. Therefore, we believe that three *OsCIN* genes—*OsCIN1*, *OsCIN4*, and *OsCIN5*—may be involved in regulating carbon metabolism so that rice plants are enabled to resist disastrous pathogen attacks.

Discussion

Identification of the rice CIN gene family

In the present study, we identified and characterized the *OsCIN* genes, members of a rice CIN gene family. Analysis of the deduced amino acid sequences predicted from *OsCIN* cDNAs revealed that they all contain the highly conserved β-fructosidase motifs and cysteine catalytic sites. These genes possess a proline in the fifth residue of the cysteine catalytic site, the presence of which is a reliable, distinctive indicator of the CINs (Goetz and Roitsch 1999). Therefore, we conclude that the genes isolated here are of the rice CIN type.

Our phylogenetic tree revealed that plant CIN are classified into four groups: monocot-I, monocot-II, dicot-I, and dicot-II. This suggests that each of the two types of monocot or dicot plants were delineated early, prior to the monocot–dicot divergence.

We used the sequenced BAC/PAC clones to locate the *OsCIN* genes on the rice chromosomes, and observed a number of transposable elements within the BAC/PAC clones encompassing these genes (data not shown). From this we could infer that the regions surrounding the *OsCIN* genes are the preferred target loci of transposable elements.

Diverse possible functions of *OsCIN* genes

In the profile analysis, we identified four genes—*OsCIN1*, *OsCIN2*, *OsCIN4*, and *OsCIN7*—that are expressed in immature seeds and which may have a role in carbon metabolism during seed development. Among them, *OsCIN1*, *OsCIN4*, and *OsCIN7* were expressed in the seed coats, while *OsCIN2* was expressed in both endosperms and seed coats. Our temporal examination revealed that these four genes were highly expressed in the ovaries or immature seeds, e.g. at 1–2 DAF (*OsCIN1*), 1–4 DAF (*OsCIN2*), and 9–15 DAF (*OsCIN7*). In addition, the high level

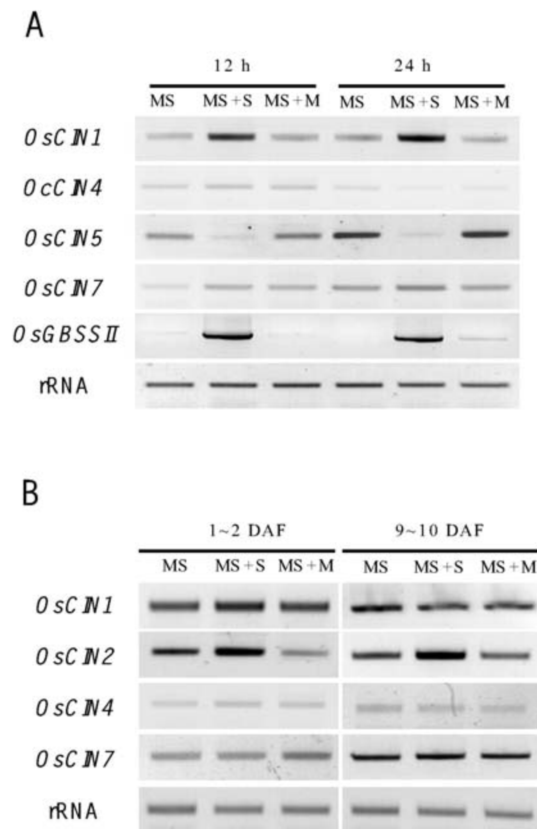

Fig. 6 Effects of sucrose on expression of *OsCIN* genes. Total RNAs collected after 12 and 24 h were used in the PCR reaction. **a** RT–PCR analysis of excised leaves supplied with sugars. **b** RT–PCR analysis of rice caryopses supplied with sugars. One- to 2-DAF and 9- to 10-DAF caryopses were treated with sugars. *MS* MS media, *MS+S* MS media+175 mM sucrose, *MS+M* MS media+175 mM mannitol

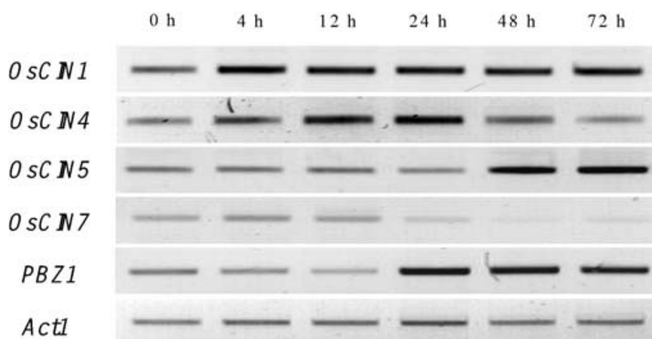

Fig. 7 Effects of blast infection on *OsCIN* gene expression. *PBZ1* and *Act1* transcripts were amplified as the PCR control. Leaves were harvested over a time course of 0–72 h

of transcript for *OsCIN2* was maintained even at 5–8 DAF. This suggests that each gene has a distinctive role in carbon partitioning during rice seed development.

Similar expression patterns have been reported for *OsCIN1* in various organs, such as the roots, sink-leaves, source-leaves, and panicles (Hirose et al. 2002). Our data were broadly consistent with those previous results, except that *OsCIN1* continued its, albeit weak, expression to 9–15 DAF. This may have been because we used a more sensitive RT–PCR analysis, rather than the Northern blot analysis conducted in that previous study. We also cannot exclude the possibility that the japonica rice genotype Jinmi tested in our study may have shown a slightly different expression during the seed development phase. As previously stated, we believe that *OsCIN1* plays an important role during the pre-storage phase, where it is involved in the proliferation of endosperm cells and the longitudinal growth of immature seeds, rather than during the starch-filling phase.

Two CINs, *HvCWINV1* and *HvCWINV2*, have been reported in barley (Weschke et al. 2003). These genes control sugar ratios in maternal and filial tissues during the early development of caryopses. In particular, *HvCWINV1* is critical for the development and differentiation of endosperm during the pre-storage phase. Its expression is found in both maternal and filial tissues, such as the endosperm and seed coat, whereas *HvCWINV2* is almost entirely seed coat-specific. *HvCWINV1* shares high homology and similar expression patterns with *OsCIN2* and *ZmIncw2* rather than with *OsCIN1*.

The maize *miniature-1* mutant, which manifests loss-of-function by the *ZmIncw2* gene, shows decreased invertase activity in the pedicel and endosperm, resulting in their aberrant development (Cheng et al. 1996; Miller and Chourey 1992). Our expression profile and phylogenetic analyses suggest that the *OsCIN2* gene plays an important role in endosperm development and longitudinal growth of seed coats, similar to the function of *HvCWINV1* and *ZmIncw2*. Its expression in both organs of rice further supports the hypothesis that *OsCIN2* shares a similar function with barley *HvCWINV1* and maize *ZmIncw2*.

Hirose et al. (2002) have reported that CIN activity is maintained at a considerably high level after 5 DAF. Here, we also demonstrated that *OsCIN2*, *OsCIN4*, and *OsCIN7* contributed, in part, to the activity of invertase after 5 DAF. In particular, *OsCIN7* was highly expressed during 9–15 DAF, suggesting that it has a major role in starch-filling, rather than pre-storage, for sucrose uptake to the endosperm during seed development.

The *OsCIN3* gene is expressed exclusively in the flowers. Such floral-specific CIN genes have also been identified in other species, such as tobacco (Goetz et al. 2001), *Arabidopsis* (Tymowska-Lalanne and Kreis 1998), and maize (Xu et al. 1996). Goetz et al. (2001) have reported that the CIN *Nin88* of tobacco has a critical role in pollen development, and that expression of the *Nin88* antisense construct induces male sterility. The importance of CIN during pollen development has been supported by the identification of an anther-specific monosaccharide transporter (Truernit et al. 1999; Ylstra et al. 1998). Therefore, we suggest that *OsCIN3* may play a role during the development of pollen with that transporter. However, it remains to be determined whether the gene is expressed specifically in rice anthers.

Regulation of *OsCIN* genes by sucrose and pathogens

Sugar, especially sucrose, serves not only as the carbon source for metabolism during plant growth and

development, but also as a signalling molecule that regulates the expression of genes involved in photosynthesis and heterotrophic metabolism (Koch 1996; Roitsch 1999; Sheen et al. 1999). Induction of CIN genes by sucrose has been described for several species, including tobacco (Krausgrill et al. 1996), Arabidopsis (Tymowska-Lalanne and Kreis 1998), and tomato (Godt and Roitsch 1997). In the present study, the expression level of *OsCIN2* was significantly increased when caryopses were treated with sucrose, which leads us to believe that this gene has a function in carbohydrate partitioning between source and sink organs as well as in establishing metabolic sink strength during seed development. In contrast, *OsCIN1* was only slightly increased in sucrose-treated, excised leaves, while *OsCIN2* showed no sucrose-inducible expression in leaf samples (data not shown). Therefore, because transcript was not present in our untreated rice leaves, it is unlikely that *OsCIN2* has a role in that organ.

Expression of CIN genes is markedly increased by wounding (Matsushita and Uritani 1974) or by infection with fungi (Benhamou et al. 1991; Hall and Williams 2000), bacteria (Sturm and Chrispeels 1990), or viruses (Herbers et al. 2000). Among the EST clones detected in our rice blast-infected leaves, we noted that some contained partial sequences corresponding to *OsCIN1* and *OsCIN4*. Rice blast, caused by *Magnaporthe grisea*, is one of the most destructive rice fungal diseases. Here, we identified three genes—*OsCIN1*, *OsCIN4*, and *OsCIN5*—whose expression was enhanced after inoculation. This suggests that these genes, especially *OsCIN1* and *OsCIN4* (which responded within 4 h of treatment), may be directly involved in the plant–pathogen interaction of switching from a housekeeping to a defence metabolism. The role of CINs in the plant–pathogen interaction remains to be unravelled. Recent studies have reported that sugar transporters play an important role in certain plant–microorganism interactions to fulfil an increased demand for carbohydrate by cells under various biotic or abiotic stress conditions (Hall and Williams 2000; Williams et al. 2000). Therefore, together with sugar transporters, CINs may help increase carbohydrate import into infected tissues.

In summary, we have now isolated *OsCIN* genes that are expressed in various rice tissues and which respond in unique patterns to sucrose and fungal stressors. We believe that these genes may function in the regulation of metabolism, growth, development, and stress responses. Therefore, it will be invaluable to determine what roles they play in gain-of-function and loss-of-function in rice plants.

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