Introduction

Phytoalexins are plant-producing antimicrobial compounds induced by both biotic and abiotic stresses, and leguminous plants mainly produce pterocarpan-based isoflavanoids as phytoalexins (Aoki et al. 2000; Ingham 1982). Pterocarpans contain two asymmetric carbons at C-6a and C-11a, but only two cis configurations are sterically possible and found in nature (Figure 1) (Dewick 1986). All levorotatory pterocarpans are widely accepted to have the (6aR,11aR) configuration, such as (−)-medicarpin (6a) and (−)-maackiain (6b), and dextrorotatory pterocarpans have the opposite configuration, such as (+)-maackiain (7a) and (+)-pterocaprin (7c) (Slade et al. 2005). Among the two enantiomers of pterocarpans, most legumes produce (−)-type enantiomers, and only a limited number of plant species, such as peanut (Arachis hypogaea), Japanese pagoda tree (Styphnolobium japonicum), and pea (Pisum sativum), produce (+)-pterocarpans (Ingham 1979; Strange et al. 1985; VanEtten et al. 1989). The stereochemistry of pterocapran is important because it determines the antimicrobial activity against pathogens; that is, some plant pathogens can detoxify (−)-isomers but not (+)-isomers, and as a result, (+)-pterocarpans show higher activity than the (−)-isomers (Delserone et al. 1992). The entire biosynthesis of (−)-pterocapran was revealed by identifying pterocapran synthase (PTS) as the long-standing missing link (Uchida et al. 2017), but further studies are required to elucidate the biosynthesis of (+)-pterocapran.

(+)−Pisatin (7c), which was the first chemically biosynthesized (+)-pterocapran-type enantiomer, has significantly higher activity than the (−)-isomer (Slade et al. 2005). However, the further development of this research was curtailed by the difficulty of obtaining the (+)-enantiomer. Therefore, the discovery of a dirigent domain-containing pterocapran synthase (PTS) is the key advance in the pterocapran research field. This enzyme catalyzes the dehydration reaction of the pterocapran skeleton to produce (+)-pterocapran (Delserone et al. 1991). A dirigent (DIR) domain is a conserved sequence found in the cytosolic domain of several protein kinases (Kumagai et al. 2001; Prat et al. 2002), and it is a key enzyme involved in the dehydration reaction. The enzymatic activity of (DIR) is highly specific to the stereochemistry of the substrate (Bolte et al. 1992), and the reaction proceeds in an enantioselective manner.

Among the protease and protein kinase families, dirigents are known to belong to the family of protein kinases (Arora et al. 1996; Bolte et al. 1992). The dirigent domain of a dirigent protein has a conserved sequence named “DIR” (Kumagai et al. 2001). The DIR domain is highly conserved in multiple pterocapran bioactive compounds; however, it is still unknown whether the DIR domain is necessary for pterocapran biosynthesis as a protein. In this study, we screened PTS homologs based on RNA-sequence data from (7c)-pisatin-producing pea seedlings and demonstrated that one of the candidates encodes isoflav-3-ene synthase (I3S). Real-time PCR analysis revealed that transcripts of I3S, in addition to other genes involved in the (+)-pisatin pathway, transiently accumulated in pea upon elicitation prior to the maximum accumulation of (+)-pisatin. I3S orthologs were also found in soybean and Lotus japonicus that are not known to accumulate (+)-pterocapran, and the catalytic function of gene products was verified to be I3S by the in vitro enzyme assay. Incubation of the crude extract of elicited soybean cells with isoflav-3-ene yielded coumestrol, suggesting that isoflav-3-ene is a precursor of coumestrol biosynthesis in soybean.
Isoflav-3-ene synthase of Pisum sativum

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identified phytoalexin and is exclusively produced by pea (Cruickshank and Perrin 1960), is one of the best-studied (+)-pterocarpan stereospecific intermediates, namely (3R)-2′-hydroxyisoflavanone and (3R,4R)-2′-hydroxyisoflavanol, the latter of which is converted to (+)-pterocarpan by PTS (Figure 1). Thus, clarifying the branching point to produce opposite stereoisomers is crucial for the elucidation of (+)-pterocarpan biosynthesis. As isoflavone reductase (IFR) catalyzes the first introduction step of chirality in pterocarpan biosynthesis, it was originally postulated to be the pivotal enzyme that determines the stereochemistry of the end product (Banks and Dewick 1982a, b). Indeed, several IFRs of the (−)-pterocarpan-producing legumes, such as soybean (Glycine max), alfalfa (Medicago sativa), and chickpea (Cicer arietinum), converted achiral 2′-hydroxyisoflavones into (3R)-2′-hydroxyisoflavonanes (Fischer et al. 1990; Paiva et al. 1991; Tiemann et al. 1991; Uchida et al. 2017). An IFR isolated from (+)-pisatin-producing tissue of pea did not produce the expected (3S)-2′-hydroxyisoflavanone and yielded only (3R)-enantiotomer (Paiva et al. 1994). The involvement of an epimerase, which converts (3R)-sophorol into (3S)-sophorol, has been previously suggested (Dewick 1986; Paiva et al. 1994). However, in a tracer experiment, 3H-labeled (3R)-sophorol was efficiently incorporated into (+)-pisatin compared with that from (3S)-sophorol (DiCenzo and VanEtten 2006), showing no evidence to support the hypothetical epimerase.

The next step to IFR is the conversion of (3R)-2′-hydroxyisoflavonane to (3R,4R)-2′-hydroxyisoflavonane 4-reductase (I4R) mediates this reaction. I4R is also designated as a sophorol reductase (SOR) in pea (DiCenzo and VanEtten 2006) and vestitone reductase (VR) in alfalfa (Guo...
results indicate that \((+)-pisatin\) \((Kaimoyo and VanEtten 2008). Taken together, these to a decrease in the \((+)-pisatin\). \(7c\) accumulation \((Kaimoyo and VanEtten 2008). Taken together, these to a decrease in the \((+)-pisatin\) accumulation. Moreover, metabolic correlation between the consumption of isoflav-3-ene and production of coumestrol was also observed in the in vitro assay using crude extract of elicited soybean cells. The results achieved herein will offer a new perspective for the elucidation of stereoisomer-specific \((+)-pterocarpan biosynthesis, mediated by DIR domain-containing proteins that produce isoflav-3-ene.

**Materials and methods**

**Chemicals**

Formononetin \((1a)\) and coumestrol \((8)\) were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Sigma-Aldrich \((St. Louis, MO, USA)\), respectively. \((\pm)-Maackaiain and \((3R,4R)-DMDI (4b)\) were kindly donated by Dr. VanEtten HD \((University of Arizona)\). DMDIF \((5b)\) was prepared from maackain according to a previous report \((Martin and Dewick 1980). \((3R,4R)-7,2′-Dihydroxy-4′-methoxyisoflavanol \((DMI, \(4a)\) was obtained from laboratory stock \((Uchida et al. 2017).\)

\((+)-Pisatin \((7c)\) was isolated from elicited pea sprouts. Pea sprouts purchased from a grocery store were soaked in 10 mM CuCl\(_2\) for 1 h and rinsed with distilled water three times. After 24 h incubation at 25°C, leaves and stems were collected and soaked in hexane. Subsequently, the hexane extracts were evaporated, and \((+)-pisatin \((7c)\) was purified by successive silica-gel thin-layer chromatography \((TLC, Silica-gel F254, Merck, Darmstadt, Germany)\) developed with the solvents, toluene : ethyl acetate=3:1 \((v/v)\) and toluene : ethyl acetate : methanol : light petroleum \((6:4:1:3, v/v/v/v)\). The nuclear magnetic resonance \((NMR)\) spectra were recorded on an ECA-500 system \((JEOL, Tokyo, Japan)\).

**Plant materials and elicitation**

Elicitation of pea seedlings was carried out according to a previous report with slight modifications \((DiCenzo and VanEtten 2006). Briefly, surface-sterilized pea seeds \((Pisum sativum cv. Usui, Takii Seed, Kyoto, Japan)\) were germinated on moist vermiculite and grown for 6 days in the dark. Cotyledons excised from seedlings were placed on filter paper soaked with 5 mM CuCl\(_2\) and periodically harvested. For the control, cotyledons were soaked with sterile distilled water. Elicited and control pea cotyledons were extracted with methanol, and the extracts were analyzed by HPLC using the conditions defined as ‘program 1’ \((Supplementary Data S1)\). The concentration of \((+)-pisatin \((7c)\) was calculated from the area under the curve of the peak of the compound.

**RNA-sequence and de novo assembly**

Total RNAs were isolated from elicited \((10 mM \text{CuCl}_2\) for 24 h) pea seedlings using the \(SV\) Total RNA Isolation System \((Promega, Madison, WI, USA)\). RNA quality was evaluated on an Agilent Bioanalyzer 2100 \((Agilent Technologies, Palo Alto, CA)\).
CA, USA). The cDNA library was prepared using the TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA, USA) and subjected to sequencing on the Illumina Miseq platform with 150 bp paired-end reads. Reads were de novo assembled with CLC Genomics Workbench ver 5.5 (CLC bio, Aarhus, Denmark) to obtain 47,799 contigs.

cDNA cloning and vector construction
cDNAs were synthesized from the total RNAs of elicited pea cotyledon using a SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). The coding sequences of PsI3F (DDBJ accession no. AAB31368) and PsSOR (DDBJ accession no. AF107404), and N-terminus truncated PsI3S1, LjI3S1, LjI3S2, and GmI3S1 were amplified and cloned into the vectors (pCR8/GW/TOPO for PsI3F, PsSOR, and PsI3S1; pET46 Ek/Lic for LjI3S1, LjI3S2, and GmI3S1) using PrimeSTAR HS DNA polymerase (TaKaRa, Shiga, Japan), the primer sets (Supplementary Data S2), and the cDNA templates prepared from elicited cotyledons or L. japonicus and soybean cDNAs (Uchida et al. 2015). A multiple expression vector was constructed using the In-Fusion cloning system (TaKaRa). Briefly, coding regions of PsI3F, PsSOR, and N-terminus truncated PsI3S1 cDNAs were transferred into the pET53-DEST vector using a Gateway System (Invitrogen). T7 promoter and coding region of PsSOR and PsI3S1 in pET53-DEST were amplified by PCR, and the PCR products were cloned into a downstream region of PsI3F cDNA in pET53-DEST.

The nucleotide sequence data reported herein have been deposited in the DDBJ, GenBank, and EMBL databases under the following accession numbers: PsI3S1, LC497416; GmI3S1, LC497417; LjI3S2, LC497419; LjI3S1, LC497418; and GmI4R, LC497420.

Heterologous expression and in vitro enzyme assay
The E. coli strain BL21-CodonPlus (DE3)-RIPL (Agilent Technologies) harboring an expression vector integrated with N-terminus truncated I3S (PsI3S1, LjI3S1, LjI3S2, and GmI3S1), were cultured overnight in 10 ml Luria Broth (LB) liquid medium containing 50 mg l−1 carbenicillin and 30 mg l−1 chloramphenicol. The cells were collected by centrifugation (9,000 g) for 1 min at 4°C, and the supernatant was extracted with methanol : light petroleum (6 : 4 : 1 : 3, v/v/v/v) and the product (Rf value = 0.49) was subsequently collected.

Polyclonal antibodies were produced using a mixture of recombinant I3S proteins (PsI3S1, 0.49 mg; GmI3S1, 0.1 mg; LjI3S1 and LjI3S2, 0.1 mg) for 10 min. Reaction mixtures were extracted with ethyl acetate and analyzed by HPLC using the conditions defined as ‘program 2’ (Supplementary Data S1).

Phylogenetic analysis
Phylogenetic analysis was performed using MEGA6 software (Tamura et al. 2013). Amino acid sequences were aligned using ClustalW, and the phylogenetic tree was constructed with the default settings of neighbor-joining method with 1,000 bootstrap replicates.

Results
RNA-sequence analysis and selection of the I3S candidate gene
RNA-sequence and de novo assemble generated 47,799 contigs. When TBlastN was employed using GeP5T1 as the query, 5 contigs that share >50% identity were obtained (Supplementary Figure S1). Among them, Psm21_contig00001700 had the highest reads per kilobase of exon model per million mapped reads (RPKM) value and seemed to contain the full-length coding sequence. The corresponding cDNA was cloned by RT-PCR and tentatively referred to as PsI3S1 for the high structural similarity (56% amino acid identity) to GeP5T1.
**Biochemical analysis of PsI3S1**

PsI3S1 was predicted to have the coding sequence of 657 bp and encode 218 amino acids. Most of the DIR domain-containing proteins have been reported to contain a putative signal peptide sequence at the N-terminus and processed into mature active forms after truncation of the signal peptide (Kim et al. 2002). The prediction of subcellular localization using WoLFPSORT (http://wolfpsort.org/) indicated that PsI3S1 is extracellular and/or vacuolar localized as well as GePTS1 (Uchida et al. 2017). Therefore, for biochemical analysis of PsI3S1, recombinant proteins were expressed as signal peptide-truncated and histidine-tagged forms in _E. coli_ cells. Enzymatic properties were examined after affinity purification, and the assay was carried out with (3R,4R)-DMDI (4b) and (3R,4R)-DMI (4a) as substrates. The substrates are unstable and rapidly convert to pterocarpan under acidified conditions; therefore, 0.1 M potassium phosphate buffer (pH 6.5), which is the same buffer as in the PTS reaction, was used for the enzyme assay (Uchida et al. 2017). The incubation mixture of PsI3S1 with (3R,4R)-DMDI (4b) or (3R,4R)-DMI (4a) gave a single product on HPLC, and the products were identified as DMDIF (5b) and 7,2′-dihydroxy-4′-methoxyisoflav-3-ene (DMI, 5a), respectively, by comparing UV, mass, or 1H-NMR spectra (Figure 2, Supplementary Figure S2, Data S3). These results show that PsI3S1 possesses isoflav-3-ene synthetic activity in vitro.

**Distribution of I3S in leguminous plants and phylogenetic analysis**

To examine the distribution of the I3S protein in leguminous plants, I3S-like cDNAs were cloned from soybean and _L. japonicus_ by RT-PCR. N-terminus truncated proteins were expressed in _E. coli_, and catalytic activity was tested using crude protein extracts with truncated proteins were expressed in _E. coli_, and catalytic activity was tested using crude protein extracts with (3R,4R)-DMI (4a) as the substrate. As a result, the I3S activity of three orthologues, GmPTS-L4 from soybean and LjPTS-L1 and LjPTS-L2 from _L. japonicus_, were confirmed (designated as GmI3S1, LjI3S2, and LjI3S1, respectively) (Figure 2). The specific activity of I3S proteins toward (3R,4R)-DMI (4a) was compared using the purified recombinant proteins. GmI3S1 showed the highest specific activity (151.6±0.8 μmol min⁻¹ mg⁻¹; relative activity, 100%). PsI3S1, LjI3S1, and LjI3S2 showed 5%, 46%, and 53% of the activity of GmI3S1, respectively. The pH dependence of I3S activity was also examined using GmI3S1 and (3R,4R)-DMI. The optimum pH range was pH 6.0–8.0, and activity decreased rapidly outside this range (Supplementary Figure S3).

The identity of amino acid sequences among I3S proteins was 55–87% (Supplementary Data S4). Alignment of the amino acid sequence of the function-

**Time courses of (+)-pisatin-accumulation and transcript levels of the biosynthetic genes in elicited pea cotyledon**

To verify whether _PsI3S1_ participates in (+)-pisatin biosynthesis, the accumulation of (+)-pisatin (7c) and the expression of its biosynthetic genes were analyzed. An abiotic stress agent, CuCl₂, activates isoflavonoid metabolism in leguminous plants (Dewick 1986). (+)-Pisatin (7c) production was induced by 5 mM CuCl₂.
Figure 3. Alignment of amino acid sequences and the phylogenetic relationships among I3S, PTS, and the related DIR domain-containing proteins. (A) The amino acid residues with at least four identical sequences are in the reverse type. Gaps (−) are inserted to optimize alignment. (B) Evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees where the associated taxa are clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Accession numbers of proteins are listed in Supplementary Data S6.

Figure 4. Time course of (+)-pisatin accumulation (A) and transcript levels of the biosynthetic genes (B) in pea cotyledon upon CuCl₂ treatment. Data are expressed as mean±SE (n=3 biological replicates). Transcript levels were analyzed using the ΔΔCt method. β-Tubulin was used as the internal standard. Transcript levels were normalized to those of non-treated cotyledons (at 0 h).

Treatment, and its maximum content was observed at 24 h and 48 h after elicitation (Figure 4A). Although (+)-pisatin (7c) was also induced by the mock treatment, its amount was more than 10-fold lower than that of the CuCl₂-treated cotyledon. The transcript levels of PsI3S1 and (+)-pisatin biosynthetic genes (PsIFS and PsIFR) were markedly increased by the elicitation, and the levels were 4.8–7.5-fold higher than the mock-treated cotyledon (Figure 4B).

One-pot synthesis of isoflav-3-ene from isoflavone using a co-cultured recombinant E. coli system and biosynthesis of coumestrol in elicited soybean cells

Isoflav-3-enes are considered to be precursors in some of the isoflavonoid pathways such as (+)-pterocarp, coumestan, and 2-arylbenzofuran. An effective method for isoflav-3-ene production is thus important to elucidate the biosynthetic mechanism. For the biotechnological production of isoflav-3-ene, recombinant E. coli cells co-expressing isoflavone 2′-hydroxylase (I2′H), cytochrome P450 reductase
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(CPR), IFR, I4R, and I3S were tested for their in vivo metabolism (bioconversion) from exogenously supplied isoflavone. First, we constructed a multiple expression vector by introducing coding sequences of PsIFR, PsSOR, and PsI3S1 of pea (Figure 5A). Recombinant E. coli cells harboring LjCPR1 and codon-optimized LjI2′H (Uchida et al. 2015) of L. japonicus or harboring PsIFR, PsSOR, and PsI3S1 were pre-cultured separately in LB liquid medium, and then both cultures were combined in one flask and fermented for 72 h in the presence of the substrate formononetin (1). As shown in Figure 5B, a new peak was observed by HPLC, but no other biosynthetic intermediates were detected in this condition. The chemical structure of the product was confirmed by 1H-NMR to be DMIF (5a). The amount of DMIF (5a) calculated from the peak area on HPLC was ca. 15 mg l−1 after 72 h of fermentation. The stability of DMIF (5a) in the neutral buffer (pH 6.5 at 30°C) was also examined. The compound was decreased by a first-order reaction, and the half-life period under this condition was 136 h.

To examine the role of I3S proteins in soybean and L. japonicus, the metabolism of isoflav-3-ene in soybean was analyzed by an in vitro enzyme assay. The substrate, 7,2′,4′-trihydroxyisoflav-3-ene (THIF, 5c), was prepared using co-cultured E. coli cells expressing I2′H (CYP81E18), IFR, I4R, and GmI3S1 of soybean and LjCPR1 of L. japonicus, with daidzein (1c) as the precursor (Supplementary Figure S4 and Data S5). Then, THIF (5c) was incubated with crude extract of elicited soybean cells, and the reaction mixture was analyzed by HPLC. As the substrate was labile, a contaminant emerged during the incubation of both the control and crude extract of soybean, but two new peaks appeared in the crude extract of soybean (Supplementary Figure S5). Based on the retention times and UV spectra, one of the peaks was identified to be coumestrol (8).

Discussion

Isoflav-3-enes have been of interest because of their characteristic biological activities, and considerably attracted attention as a key intermediate in (+)-pisatin biosynthesis of pea. In the present study, we identified I3S cDNAs from pea (PsI3S1), soybean (GmI3S1), and L. japonicus (LjI3S1 and LjI3S2). It was predicted that all I3S proteins contained a putative signal peptide sequence at the N-terminus and were localized to extracellular region or vacuole. Although their subcellular localizations were not experimentally verified, the signal peptide-truncated proteins were found to produce only isoflav-3-enes from (3R,4R)-2′-hydroxyisoflavonols and functionally distinct from PTS proteins. The specific activities of the soybean and L. japonicus I3S proteins for (3R,4R)-DMI (4a) were roughly at the same level as that of recombinant PTS proteins, but PsI3S1 showed lower activity than the other I3S proteins (Uchida et al. 2017). (+)-Pisatin (7c) has a methylenedioxy ring, and the bridge formation is thought to occur at the early stage of biosynthesis because 14C-labeled methylenedioxylated isoflavone (pseudobaptigenin, 1b) was efficiently incorporated into pisatin (Banks and Dewick 1982a) (Figure 1). Thus, it is assumed that the natural substrate of PsI3S1 is (3R,4R)-DMDI (4b) (Celoy and VanEtten 2014), and the low activity of PsI3S1 toward (3R,4R)-DMI (4a) is attributable to the substrate preference of the protein.

Figure 5. One-pot synthesis of isoflav-3-ene from isoflavone. Multiple expression vector and biosynthetic scheme (A) and HPLC chromatogram (B) are shown. Upper chromatogram and lower chromatogram show 0 h and 72 h after incubation with 1a, respectively (1a: formononetin, 5a: DMIF). T7 Pro, T7 promoter; T7 Ter, T7 terminator; AmpR, ampicillin resistance gene.
In general, the biosynthetic genes of phytoalexins are induced by biotic and abiotic stresses (e.g., attack by pathogens, herbivory, and elicitor treatment), and are considerably upregulated prior to the increased accumulation of phytoalexins. Therefore, comparing the time courses of (+)-pisatin (7c) accumulation and transcript levels is helpful to estimate the relevant biosynthetic pathway. In the present study, transient upregulation of PsI3S1, PsIFS, and PsIFR transcripts was clearly observed, compared to non-elicited, in pea cotyledon prior to (+)-pisatin (7c) accumulation (Figure 4). These results strongly support the possibility that PsI3S1 is involved in (+)-pisatin biosynthesis. The external stimuli, such as mechanical wounding, induce phytoalexins accumulation. (+)-Pisatin (7c) production was also induced by water (mock) treatment, even though the amount was over 10-fold lower than that of elicitor-treated cotyledons. This result indicates that cutting cotyledons from seedlings promoted (+)-pisatin (7c) accumulation.

Generally, structures of phytoalexin are lineagespecific, and (+)-pisatin (7c) is a characteristic product (namely, specialized metabolite) in pea. Most leguminous plants such as soybean and L. japonicus produce (-)-terocarpan and their derivatives by stress responses, whereas the biosynthesis of (+)-terocarpan remains unknown, and biosynthetic mechanisms of coumestan and 2-arylbenzofuran have also been a matter of debate (Kinoshita 1997; Martin and Dewick 1980). Considering that coumestrol and its derivatives are widely distributed among leguminous plants, and soybean cells produce a prenylated coumestrol by elicitation (Yoneyama et al. 2016), our data suggest that the soybean and L. japonicus I3S proteins participate in coumestan biosynthesis in planta. In fact, isoflavones were efficiently converted into the end product isoflav-3-ene (Figure 5). DMIF (5a) could be a characteristic product (namely, specialized metabolite) in pea. Most leguminous plants such as soybean and L. japonicus produce (-)-terocarpan and their derivatives by stress responses, whereas the biosynthesis of (+)-terocarpan remains unknown, and biosynthetic mechanisms of coumestan and 2-arylbenzofuran have also been a matter of debate (Kinoshita 1997; Martin and Dewick 1980). Considering that coumestrol and its derivatives are widely distributed among leguminous plants, and soybean cells produce a prenylated coumestrol by elicitation (Yoneyama et al. 2016), our data suggest that the soybean and L. japonicus I3S proteins participate in coumestan biosynthesis in planta. In fact, isoflavones were efficiently converted into the end product isoflav-3-ene (Figure 5). DMIF (5a) could be
recovered at a relatively high yield (ca. 80%) by silica-gel TLC, whereas THIF (5c) was unstable and easily decomposed on silica-gel TLC (recovery, ca. 20%). In the present study, TLC-purified THIF (5c) was used for the biosynthetic analysis of coumestrol, but we recently found that the degradation and recovery (ca. 70%) of the compound can be improved by using C18 octadecylsilyl column chromatography with aqueous methanol solution as the solvent (data not shown). Preparation of isoflav-3-ene by this system will therefore accelerate the elucidation of the biosynthetic mechanism introducing (+)-chirality in pea as well as coumestrol biosynthesis in soybean.

Acknowledgements

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