Biochemical and Molecular Characterization of RcSUS1, a Cytosolic Sucrose Synthase Phosphorylated in Vivo at Serine 11 in Developing Castor Oil Seeds*

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Background: The pathways and control of oil seed sugar unloading and metabolism are not well understood.

Results: The UDP-specific sucrose synthase isozyme RcSUS1 is the dominant sucrolytic enzyme of developing castor oil seeds (COS) and identified as RcSUS1 by mass spectrometry. RcSUS1 transcripts peaked during early development, whereas levels of SUS activity and immunoreactive 93-kDa SUS polypeptides maximized during mid-development, becoming undetectable in fully mature COS. The cytosolic location of the enzyme was established following transient expression of RcSUS1-enhanced YFP in tobacco suspension cells and fluorescence microscopy. Immunological studies using anti-phosphosite-specific antibodies revealed dynamic and high stoichiometric in vivo phosphorylation of RcSUS1 at its conserved Ser-11 residue during COS development. Incorporation of [γ-32P]ATP into a RcSUS1 peptide substrate, alongside a phosphosite-specific ELISA assay, established the presence of calcium-dependent RcSUS1 (Ser-11) kinase activity. Approximately 10% of RcSUS1 was associated with COS microsomal membranes and was hypophosphorylated relative to the remainder of RcSUS1 that partitioned into the soluble, cytosolic fraction. Elimination of sucrose supply caused by excision of intact pods of developing COS abolished RcSUS1 transcription while triggering the progressive dephosphorylation of RcSUS1 in planta. This did not influence the proportion of RcSUS1 associated with microsomal membranes but instead correlated with a subsequent marked decline in SUS activity and immunoreactive RcSUS1 polypeptides. Phosphorylation at Ser-11 appears to protect RcSUS1 from proteolysis, rather than influence its kinetic properties or partitioning between the soluble cytosol and microsomal membranes.

In developing seeds, the partitioning of imported photosynthetic carbon into starch, storage lipid, and storage protein biosynthesis is of considerable agronomic interest because seeds are the major source of plant-derived nutrients for worldwide food and feed industries. Seed development requires a large influx of carbon and energy in the form of sucrose, the major type of photosynthetically assimilated carbon translocated from source leaves to sinks via the phloem. Imported sucrose must be enzymatically cleaved into hexoses as an initial step in the biosynthesis of seed storage products, namely starch, protein, and triacylglycerides (oil). Sucrose cleavage is vital for vascular tissues such as developing seeds or tubers in which SUS has been considered to be predominantly involved in supporting polysaccharide (starch and/or cell wall) biosynthesis (1, 3). However, transcriptomic and proteomic studies, together with enzyme activity assays and immunolocalization indicate that SUS also fulfills an important function in developing oil seeds to support the production of carbon skeletons and reducing power (via glycolysis) required for triacylglyceride and storage protein synthesis (4–9). There is direct evidence of SUS importance in seeds of several crop plants, in which a reduction in

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‡ The abbreviations used are: CDPK, calcium-dependent protein kinase; COS, castor oil seed (R. communis); EYFP, enhanced YFP; PEP, phosphoenolpyruvate; LTQ-FT MS/MS, linear ion-trap Fourier transform tandem mass spectrometer; qPCR, quantitative real time PCR; SUS, sucrose synthase.
The metabolism of imported photosynthate by developing seeds, the genetic origin, biochemical properties, and in vivo phosphorylation status of native oil seed SUS isozymes are poorly understood. Herein, we describe the molecular and biochemical properties of SUS from castor (Ricinus communis) oil seeds (COS). COS is a model heterotrophic (non-green) oil seed that contains up to 60% (by weight) storage triacylglycerides at maturity, as compared with ~20 and 40% in the photoautotrophic oil seeds soybean (Glycine max) and rapeseed (Brassica napus), respectively. Production of phosphorylation state- and site-specific antibodies against the conserved N-terminal seryl phosphorylation site of the enzyme allowed us to study in detail changes in SUS phosphorylation status as a function of seed development and photosynthesize supply.

**EXPERIMENTAL PROCEDURES**

**Plant Material**—Castor bean plants (R. communis; cv. Baker 296) were cultivated in a greenhouse at 24 °C and 70% humidity under natural light supplemented with 16 h of artificial light. Pods containing developing COS at heart-shaped embryo (stage III), mid-cotyledon (stage V), full cotyledon (stage VII), and maturation (stage IX) stages of development (32) were harvested at midday unless otherwise indicated, and endosperm and cotyledon tissues were rapidly dissected. For depodding treatments, stems containing intact pods of developing COS were excised and placed in water in the dark at 24 °C. Gerninated COS was obtained as previously described (33). All tissues were frozen in liquid N₂ and stored at −80 °C until used.

**Bioinformatics, RT-PCR, and qPCR**—Castor SUS (RcSUS) genes were identified with BLASTP using known Arabidopsis SUSs as queries, whereas amino acid sequence alignments were performed using ClustalX (version 1.81). Total RNA was extracted and purified as described previously (33). RNA samples were evaluated for purity via their A₂₆₀/A₂₈₀ ratio and integrity by resolving 1 μg of RNA on a 1% (w/v) denaturing agarose gel. RNA (5 μg) was reverse transcribed with Superscript III (Invitrogen), and semiquantitative RT-PCR or qPCR accomplished using gene specific primers (Table 1) that were designed using Primer3Plus or DNAMAN software (version 5.0). All PCR products were verified by sequencing. RcACTIN (AY360221) was used as an internal control for normalization. Conditions were optimized for all RT-PCRs to ensure linearity of response for comparison between samples. Primer pairs yielded fragments of the expected size. Control RT-PCRs lacking reverse transcriptase did not show any bands.

An Applied Biosystems 7500 real time PCR system and iTaq™ Universal SYBR® Green Supermix (Bio-Rad) were used for qPCR. The reaction conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. The data were analyzed with Applied Biosystems 7500 software (version 2.0.1). RsSUS1 expression was measured by using the absolute quantification method (35). All qPCR experiments were repeated at least three times using cDNAs prepared from two samples.

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**FIGURE 1.** Model highlighting metabolic functions of SUS in developing seeds. SUS is the dominant sucrolytic enzyme in developing seeds. Its products are channeled into starch, cellulose, or fatty acid biosynthetic pathways or the Krebs’ cycle either for ATP production via oxidative phosphorylation or for the anaplerotic replenishment of intermediates withdrawn for biosynthesis. P, phosphate.
**In Vivo Phosphorylation of Castor Bean RcSUS1**

### TABLE 1

| Experimental procedure | Gene       | Primer designation | Sequence (5’ → 3’) |
|------------------------|------------|--------------------|-------------------|
| RT-PCR                 | RcSUS1     | RcSUS1-F           | TGAAGAACATTCTGAAGTATGGACCG |
|                        | RcSUS1-R   | TGAAGAAGCTCTTGCCTTACCA |
|                        | RsSUS2-F   | CGGGAACCTTGCTGCTCGAACATG |
|                        | RsSUS2-R   | CGGGAACCTTGCTGCTTGAGAAG |
|                        | RsSUS3-F   | TGGCTAGCTGAGCTGCTTCTC |
|                        | RsSUS3-R   | AGGCTCCCTCTTGCTGCTGCTC |
|                        | RsSUS4-F   | TCCCTGAGAATACCCACCG |
|                        | RsSUS4-R   | ACCCTCTGAACACCCCGACA |
|                        | RsSUS5-F   | CCTTTTGCTGCTGCCGGG |
|                        | RsSUS5-R   | ACCCAGGCTGAGCAGCACTTCT |
|                        | RsActin-F  | GTCAATCCGCTCCGTCGGAAG |
|                        | RsActin-R  | GTCATATCTCACCTCTGAGAAA |
| qPCR                   | RsSUS1-qPCR-F | ACCCTGACCTTTCCGCTA |
|                        | RsSUS1-qPCR-R | CAATGTTAAGGACTACCTCTG |
|                        | RcActin-qPCR-F | TGCTGCTGTTTTCGCCCCG |
|                        | RcActin-qPCR-R | ACTCTGATACCTCCGGGAGAAT |
| Amplifying RcSUS1      | pSAT6-EYFP-RcSUS1 | GAGAGCGATGAGACACCCCTT |
|                        | Inf-RcSUS1-EYFP-F | CGAACGATACCGCTGAGGCTGCT |
|                        | Inf-RcSUS1-EYFP-R | GTCAGATGAGACGACGAGGAAAC |

**Isolation of RcSUS1 cDNA, Construction of Plasmids, Transient Expression, and Imaging of Tobacco Suspension Cells**—A full-length RcSUS1 clone (GenBank™ accession number KJ789950) was isolated from a cDNA library prepared from stage V developing COS (33) and inserted into PET28b. RcSUS1 from pET28b-RcSUS1 was inserted into pSAT6-EYFP-C1 using Xhol and Xmal to yield RcSUS1-enhanced YFP (EYFP). Transient transformation of tobacco cells with RsSUS1-EYFP and mCherry-RcPPC3 (encoding cytosolic targeted plant-type phosphoenolpyruvate (PEP) carboxylase from developing COS) (34) was performed with 5 μg of each plasmid DNA using a Bio-Rad Biolistic particle delivery system. Bombarded cells were incubated for 8 h to allow for gene expression and protein sorting, fixed in 4% (w/v) formaldehyde, and imaged using epifluorescence microscopy as previously described (34).

**SUS Activity Assays, Kinetic Studies, and Determination of Soluble Protein Concentration**—SUS was assayed at 24 °C by following the reduction of NAD⁺ or oxidation of NADH at 340 nm using a Spectrmax Plus 340 microplate spectrophotometer (Molecular Devices) and the following assay conditions. The standard reaction mix for the forward, sucrose-coneilling direction contained 50 mM Hepes-KOH (pH 7.0), 100 mM sucrose, 1 mM UDP, 0.5 mM ATP, 0.5 mM NAD⁺, 2 units/ml yeast hexokinase, 1.5 units/ml rabbit muscle phosphoglucone isomerase, and 1 unit/ml Leucosacostis glucose-6-phosphate dehydrogenase (corrected for any contaminating invertase activity by omitting UDP from the reaction mix), and that for the reverse, sucrose-sythesizing, direction contained 50 mM Hepes-KOH (pH 7.0), 0.15 mM NADH, 10 mM fructose, 2 mM UDP-glucose, 1 mM PEP, 4 units/ml rabbit muscle pyruvate kinase, and 8 units/ml rabbit muscle lactate dehydrogenase. Coupling enzymes were desalted before use, and assays were initiated by the addition of UDP (cleavage) or UDP-glucose (synthesis). One unit of activity is defined as the amount of SUS resulting in the production of 1 μmol of product/min. All assays were linear with respect to time and concentration of enzyme assayed. Apparent Vₘₐₓ and Kₘ values were calculated using a computer enzyme kinetics program as previously described (36). All kinetic parameters are the means of a minimum of three independent experiments and are reproducible within 10% of the mean value. Metabolite stock solutions were adjusted to pH 7.0.

Protein concentrations were determined using a Coomassie Blue G-250 colorimetric method and bovine γ-globulin as the protein standard as previously described (36).

**Preparation of Clarified Extracts Used in Time Course Studies**—Quick frozen tissues were ground to a powder in liquid N₂ and homogenized (1:2, w/v) using a Brinkmann PT-3100 Polytron (Mississauga, Canada) in ice-cold buffer A, which contained 50 mM KPi (pH 7.0), 1 mM EGTA, 1 mM EDTA, 0.1% (v/v) Triton X-100, 20% (v/v) glycerol, 4% (w/v) PEG 8,000, 0.2 mM Na₂VO₄, 0.2 mM Na₂MoO₄, 1 mM NaPP, and 1% (w/v) poly(vinyl pyrrolidone). Homogenates were centrifuged at 4 °C and 15,000 × g for 10 min, and resulting clarified extracts were rapidly assayed for SUS activity and total protein or prepared for SDS-PAGE and immunoblotting.

**Buffers Used During SUS Purification**—All buffers were degassed and contained protein-phosphatase inhibitors (0.2 mM Na₂VO₄, 0.2 mM Na₂MoO₄, and 1 mM NaPP). Buffer B contained 600 mM KPi (pH 7.5). Buffer C contained 25 mM KPi (pH 7.5) and 10% (v/v) ethylene glycol. Buffer D contained 25 mM Hepes-KOH (pH 8.0), 10% (v/v) ethylene glycol, and 20% (v/v) glycerol. Buffer E contained 25 mM Hepes-KOH (pH 8.0) and 15% (v/v) glycerol.

**SUS Purification from Developing COS and Native Molecular Mass Determination**—All chromatographic steps were carried out at 24 °C using an ÄKTA Purifier FPLC (GE Healthcare). Quick frozen endosperm (75 g) from stage V–VII developing COS was homogenized in 150 ml of ice-cold buffer A as described above and centrifuged. PEG 8000 (50% (w/v) in 50 mM Hepes-KOH, pH 7.5) was added to a final concentration of 20% (w/v), and the solution was stirred for 20 min at 4 °C and centrifuged. PEG pellets were resuspended in 210 ml of buffer B to a final protein concentration of 15 mg/ml. Following centrifugation, the supernatant was loaded at 3 ml/min onto a column (2.2 × 10 cm) of butyl Sepharose 4 Fast Flow (GE Healthcare) equilibrated with buffer B. The column was washed until A₂₈₀ approached baseline, and SUS eluted with 190 ml of a linear gradient of a simultaneously decreasing concentration of buffer B (100–0%) and an increasing concentration of buffer C (0–100%). Pooled peak fractions were concentrated to 2 ml using an Amicon Ultra-15 centrifugal filter unit (30-kDa cutoff) and applied at 0.3 ml/min onto a Superdex 200 HiLoad 16/60
In Vivo Phosphorylation of Castor Bean RcSUS1

In Vitro Dephosphorylation of RcSUS1 and Determination of RcSUS1 Phosphorylation Stoichiometry—Clarified COS extracts or purified RcSUS1 were desalted into the standard dephosphorylation buffer (50 mM Tris-HCl, pH 7.5, containing 5 mM MgCl₂, 1 mM DTT, and 20% (v/v) glycerol) using Micro Spin-OUT GT-12000 desalting columns (Geno Technology). Incubation with λ-phosphatase (New England Biolabs) was performed as described previously (36) in 50-µl reactions containing 150 µg of protein. RcSUS1 phosphorylation stoichiometry was estimated by incubating a clarified stage III–V endosperm extract or purified RcSUS1 (that had each been preincubated with and without λ-phosphatase as described above) containing 4 milli-units of SUS activity with 25 mM Hepes-KOH (pH 7.4), 10% (v/v) glycerol, 5% (w/v) PEG 8000, 20 µg/ml dephosphopeptide, and 20 µl of anti-Ser(P)-11 immune serum in a total volume of 0.1 ml. The mixture was incubated for 1 h at 30 °C and then overnight on ice. A 10% (w/v) suspension of Staphylococcus aureus cell walls (Sigma-Aldrich) in PBS (25 µl/tube) was added. Following a 30-min incubation at 30 °C and centrifugation at 14,000 × g for 5 min, SUS cleavage activity in the supernatant was measured.

RcSUS1 Kinase Assays—The radiometric RcSUS1 kinase assay mix contained 50 mM Hepes-KOH (pH 7.4), 50 mM microcystin-LR, 1 mM DTT, 0.02% (v/v) Brij-35, butyl Sepharose-enriched stage V–VII COS endosperm extract as kinase source, 0.4 mM dephosphopeptide, 0.2 mM [γ-32P]ATP (1250 cpm/pmol), 1 mM MgCl₂, and 0.1 mM CaCl₂. Reactions were incubated for 30 min at 30 °C, and after which they were adsorbed onto 1-cm² squares of P81 filter paper. The papers were washed in 1% (v/v) H₃PO₄ for 1 h, and 32P incorporation was quantified using an LS 6500 multipurpose scintillation counter (Beckman Coulter).

Phospho-site-specific ELISA-based RcSUS1 kinase assays were performed using anti-Ser(P)-11 and medium-binding 96-well ELISA plates. Incubations took place for 30 min at 30 °C and included phosphate inhibitors (1 mM NaPPi, 0.2 mM Na₂MoO₄, and 0.2 mM Na₃VO₄). Plates were incubated with 50 µl/well of 2 µg/ml dephospho-RcSUS1 diluted in PBS and then washed twice with PBS, prior to blocking overnight at 4 °C with 5% (w/v) skim milk powder dissolved in PBS. Plates were washed four times with PBS and incubated with 100 µl/well of kinase reaction mix containing 50 mM Hepes-KOH (pH 7.4), 1 mM ATP, 1 mM DTT, 0.1 mM CaCl₂, phosphate inhibitors, and the kinase source being tested. Reactions were terminated by washing wells four times with PBS. Plates were then incubated with 100 µl/well of anti-Ser(P)-11 diluted 5,000-fold in PBS containing phosphate inhibitors, washed four times with PBS, incubated with 100 µl/well of a goat anti-rabbit IgG alkaline phosphate-conjugated secondary antibody diluted 10,000-fold in PBS containing phosphate inhibitors, and washed four times with PBS. Wells were washed with 100 mM Tris-HCl (pH 9.5) containing 100 mM NaCl and 5 mM MgCl₂ prior to adding 100 µl/well of 1 mg/ml para-nitrophosphophate dissolved in the same buffer. The rate of para-nitrophosphophate production was determined by continuously monitoring the increase in A₄₀₅ using a Spectromax Plus 340 Microplate Spectrophotometer (Molecular Devices).

Mass Spectrometry—Proteins were reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, and dialyzed against 10 mM ammonium bicarbonate. Following digestion with sequencing grade trypsin, chymotrypsin, or endoproteinase Asp-N (Roche Diagnostics), the respective peptides were dissolved in 0.2% (v/v) formic acid for analysis on a Nano-
Acquity ultraperformance liquid chromatography system (Waters) coupled to a 7-tesla hybrid linear ion trap Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FIT MS/MS; Thermo Fischer Scientific, Inc.) as previously described (37). Phosphopeptide identification was performed using an in-house Mascot Server (version 2.3.0; Matrix Science), and the data were interrogated using the NCBI database for viridiplantae. Phosphorylation sites were validated by manual inspection of MS/MS spectra with predicted fragments.

### TABLE 2

Predicted properties of deduced castor SUS isozymes and comparison of amino acid sequence identity of RcSUS1 with other members of the castor SUS family, as well as with SUS orthologs from other vascular plants

| Name       | NCBI protein accession number | Identity to RcSUS1 % | Length residues | Predicted size kDa | Predicted pI pH |
|------------|--------------------------------|----------------------|-----------------|-------------------|-----------------|
| RcSUS1     | XP_002516210                   | 100                  | 805             | 92.4              | 5.98            |
| RcSUS2     | XP_002516963                   | 68                   | 805             | 92.1              | 5.72            |
| RcSUS3     | XP_002523115                   | 70                   | 807             | 92.2              | 6.20            |
| RcSUS4     | XP_002526290                   | 56                   | 867             | 97.5              | 8.33            |
| RcSUS5     | XP_002532791                   | 55                   | 831             | 94.3              | 6.52            |
| GmSUS1<sup>a</sup> | NP_001237525                   | 88                   | 805             | 92.2              | 6.04            |
| AtSUS1<sup>d</sup> | NP_001031915                   | 84                   | 808             | 93.0              | 5.83            |
| AtSUS2<sup>d</sup> | NP_199730                      | 70                   | 807             | 92.0              | 5.70            |
| AtSUS3<sup>d</sup> | NP_192137                      | 68                   | 809             | 92.0              | 5.85            |
| AtSUS4<sup>d</sup> | NP_366865                      | 85                   | 808             | 93.0              | 6.12            |
| AtSUS5<sup>d</sup> | NP_198534                      | 53                   | 836             | 94.9              | 6.23            |

<sup>a</sup> Determined via ClustalX (version 1.81) sequence alignment.

<sup>b</sup> As computed by ExPASy prediction programs.

<sup>c</sup> Soybean (G. max) root nodule SUS (nodulin 100).

<sup>d</sup> A. thaliana SUS isozymes.

### RESULTS

**RcSUS1 Is the Dominant SUS Isozyme Expressed in Developing Castor Oil Seeds**—Interrogation of the castor genome identified five SUS genes (RcSUS1–RcSUS5) predicted to encode 92–97-kDa polypeptides sharing high (>55%) amino acid sequence identities (Table 2). Expression of the RcSUS1–5 gene family in developing COS has been previously documented in a
In Vivo Phosphorylation of Castor Bean RcSUS1

TABLE 3
Purification of RcSUS1 from 75 g of endosperm harvested from stage V–VII developing COS

| Step                | Activity | Protein | Specific activity | Purification | Yield |
|---------------------|----------|---------|-------------------|--------------|-------|
| Clarified extract   | 71 units | 3393 mg | 0.02 units/mg     | 1.0          | 100   |
| PEG fractionation   | 81 units | 1734 mg | 0.05 units/mg     | 2.2          | 113   |
| Butyl Sepharose     | 59 units | 134 mg  | 0.44 units/mg     | 21           | 83    |
| Superdex-200        | 43       | 24      | 1.8 units/mg      | 84           | 60    |
| Mono Q              | 13       | 3.6     | 3.5 units/mg      | 170          | 18    |

In the process of purifying RcSUS1 from developing COS, a single 93-kDa polypeptide (p93) was isolated, which is the dominant sucrolytic enzyme expressed in the endosperm of mature (dry) COS. The corresponding SUS activity profiles and SDS-PAGE patterns demonstrated that SUS is the dominant sucrolytic enzyme expressed in the endosperm of mature (dry) COS. The SUS activity and polypeptide profiles were consistent with the SUS activity and polypeptide profiles reported for homogeneous SUS from a range of plant sources (16, 21, 23). The SUS activity and polypeptide profiles were consistent with the SUS activity and polypeptide profiles reported for homogeneous SUS from a range of plant sources (16, 21, 23).

To assess the effect that photosynthate supply to developing COS has on RcSUS1 expression, stems containing intact pods of castor fruits were excised (depodded), placed in water, and incubated in the dark at 24 °C for 48 h. This led to a pronounced down-regulation of RcSUS1 transcripts in endosperm and cotyledon of developing COS (Fig. 2D).

SUS Activity, Subunit Composition, and Polypeptide Abundance in Developing COS—SUS is the dominant sucrolytic enzyme of developing COS as indicated by the UDP-dependent sucrose cleavage activity of clarified endosperm extracts (Fig. 3A). SUS activity was maximal during mid-development (stages V–VII) and then decreased to undetectable levels in fully mature COS. To visualize SUS polypeptide(s), an immunoblot of endosperm extracts from different stages of development was probed with anti-soybean root nodule SUS immune serum (Fig. 3B). The immunoblots uniformly cross-reacted with a single 93-kDa polypeptide (p93) whose relative abundance paralleled the corresponding SUS activity profiles. No immunoreactive SUS polypeptides were apparent on immunoblots of extracts from fully mature COS (Fig. 3B). Levels of SUS activity and immunoreactive p93 were considerably more abundant in developing versus germinating COS (Fig. 3, A and B).

The SUS of developing COS is remarkably stable in vitro, because negligible degradation of its p93 subunits or reduction in cleavage activity occurred when a clarified endosperm extract was incubated at 24 °C for up to 24 h in the absence of protease inhibitors (Fig. 3C). By contrast, other key glycolytic enzymes extracted from developing COS, such as PEP carboxylase and plastidic pyruvate kinase, are extremely prone to partial degradation by endogenous thiol protease activity (33, 42).

**SUS Purification from Developing COS and Its Identification as RcSUS1**—To assess the influence of COS development on SUS more thoroughly, 3.6 mg of SUS was purified 170-fold from 75 g of stage V–VII developing COS, with an overall recovery of 18% (Table 3). A single peak of SUS activity was resolved during all chromatography steps. The final specific activity of 3.5 units/mg compares favorably with values reported for homogeneous SUS from a range of plant sources (16, 21, 23). When the final preparation was denatured and subjected to SDS-PAGE, major 93-kDa and minor 180-kDa protein-staining polypeptides (p93 and p180, respectively) were obtained that cross-reacted with anti-soybean root nodule SUS immune serum (Fig. 4, A and B). Sequencing of endoproteinase Asp-N, trypsin, and chymotrypsin peptide digests via LTQ-FT MS/MS identified p93 as RcSUS1 (99% sequence coverage) (Fig. 5). The minor p180 present in the final preparation (Fig. 4, A and B) was also identified as RcSUS1 by mass spectrometry (results not shown). p180 appears to consist of cross-linked p93 homodimers that formed during RcSUS1 purification. When an aliquot of purified RcSUS1 was boiled in SDS sample buffer lacking thiol-reducing reagents prior to SDS-PAGE, p93 and p180 appeared to become further cross-linked into higher Mₙ species (Fig. 4C).

A native ₃₈ of 403 ± 6 kDa was estimated by gel filtration FPLC of the final preparation on a calibrated Superdex 200 column. Thus, similar to most other plant SUSs (1), native RcSUS1 from developing COS appears to exist as a homotrimer composed of identical p93 subunits.

**Cloning and Subcellular Location of RcSUS1**—A full-length RcSUS1 clone was isolated from a cDNA library prepared from stage V developing COS. The corresponding deduced 92.4-kDa polypeptide (Table 2) contains a putative seryl phosphorylation domain near its N terminus, which encompasses a basophilic domain near its N terminus, which encompasses a basophilic
kinase motif for plant CDPKs (hydrophobic-Xaa-basic-Xaa-Xaa-(Ser/Thr)) (Fig. 5, A and B). This motif is present in most plant SUS sequences reported to date (Fig. 5B), and the Ser-11 residue of RcSUS1 corresponds to the well documented in vivo seryl phosphorylation site of several nonseed SUS orthologs (1, 17–19, 21, 27–29).

To determine the subcellular location of RcSUS1, its coding region was fused with the 3′/H11032 end of an EYFP reporter gene and transiently expressed via biolistic bombardment in tobacco suspension cells under the control of the cauliflower mosaic virus 35S promoter. Epifluorescence microscopy demonstrated that RcSUS1-EYFP was targeted exclusively to the cytosol (Fig. 6), as evidenced by its co-localization with co-expressed, fluorescent protein-tagged plant-type PEP carboxylase from developing COS (mCherry-RcPPC3), a well characterized cytosolic marker fusion protein (34). This result is consistent with bioinformatics analyses of the RcSUS1 sequence, which failed to predict the presence of any membrane-spanning domains or mitochondrial or plastid targeting peptides in its N terminus.

In Vivo Phosphorylation of RcSUS1 at Ser-11 by a Calcium-dependent Protein Kinase—A Ser(P)-11 specific antibody was raised against a synthetic phosphopeptide corresponding to residues 4–18 of RcSUS1 (Fig. 7A). When incubated in the presence of 10 μg/ml of the corresponding dephosphopeptide, anti-Ser(P)-11 detected as little as 25 ng of the phosphopeptide on dot blots but did not cross-react with 200 ng of the dephosphopeptide (Fig. 7B). The cross-reaction with the phosphopeptide was largely quenched when a parallel dot blot was incubated with anti-Ser(P)-11 containing 10 μg/ml of the blocking phosphopeptide. The use of these blocking peptides alongside the inclusion of λ-phosphatase-treated RcSUS1 control lanes served to establish anti-Ser(P)-11 specificity in subsequent
In Vivo Phosphorylation of Castor Bean RcSUS1

FIGURE 7. Phosphorylation of RcSUS1 at Ser-11 in developing COS. A, sequence of synthetic P-peptide that was covalently coupled to KLH and used for rabbit immunization. The peptide was synthesized with an extra N-terminal Cys residue to facilitate its conjugation to KLH. The Ser-11 phosphorylation site is indicated. B, dot blots of varying amounts of the P-peptide and corresponding deP-peptide were probed with anti-Ser(P)-11 in the presence of 10 μg/ml of P- or corresponding deP-peptide. C, purified RcSUS1 and a clarified homogenate from stage V–VII COS were incubated at 30 °C for 30 min with (+) and without (−) λ-phosphatase (λ-Ptase) in the presence and absence of a phosphatase inhibitor mixture (0.2 mM Na3VO4, 0.2 mM Na2MoO4, 1 mM NaPPi, and 50 nM P’tase inhibitors: - + + +). Assays were initiated by the addition of 0.2 mM of [γ-32P]ATP (++) or unlabeled ATP (−−) and incubated at 30 °C for 30 min unless otherwise indicated. Assays lacking Ca2+ contained 1 mM EGTA, and a butyl Sepharose-enriched stage V–VII developing COS extract was desalted and used as the kinase source. Linearity of the radiometric assay with respect to time (E) and linearity of the ELISA-based assay with respect to amount of COS extract (F) were determined in the presence of 0.1 mM Ca2+; all values represent the mean of n = 2 independent experiments and are within ±15% of the mean value. G, I, stems containing pods of developing COS were excised and placed in water in the dark at 24 °C for up to 72 h. At various times post-depodding, endosperm was rapidly dissected from stage V–VII COS and assayed for SUS cleavage activity (G), relative amounts of immunoreactive p93 subunits (as determined by immunoblotting with anti-RcSUS1 and densitometry (H), and relative phosphorylation of p93 at Ser-11 (I) as described for D. All values in G–I represent means ± S.E. of n = 4 biological replicates. Asterisks denote values that are significantly lower (p < 0.05) than those obtained at t = 0 h. deP-peptide, dephosphopeptide; P-peptide, phosphopeptide.

immunoblots. Our use of anti-Ser(P)-11 was complemented with an antibody raised the purified RcSUS1 (anti-RcSUS1). This antibody cross-reacted with SUS polypeptides irrespective of their phospho-status, thus allowing for standardization of total RcSUS1 on immunoblots. The results presented in Fig. 7C revealed that the p93 subunit of RcSUS1 was phosphorylated at Ser-11. This was verified during LTQ-FT MS/MS analysis of purified RcSUS1, which detected a single phosphorylation site corresponding to Ser-11 (results not shown). Both anti-RcSUS1 and anti-Ser(P)-11 were monospecific for the RcSUS1 p93 subunit on immunoblots of clarified COS extracts (results not shown). The immunoblots also revealed the presence of phosphatase activity in COS extracts that effectively dephosphorylated RcSUS1 in vitro, as reflected by the marked reduction in Ser-11 phosphorylation when a clarified extract was incubated in the absence of phosphatase inhibitors and λ-phosphatase (Fig. 7C).

The stoichiometry of phosphorylation at Ser-11 was estimated by quantifying the recovery of SUS activity after anti-Ser(P)-11 was used to immunoprecipitate phospho-RcSUS1. Anti-Ser(P)-11 immunoprecipitated 100 and 70% of the total SUS activity present in a stage III–V COS extract or purified RcSUS1 preparation, respectively. By contrast, no SUS activity was immunoprecipitated by preimmune serum or when the clarified extract or purified RcSUS1 was preincubated with λ-phosphatase for 30 min, prior to the addition of the anti-
In Vivo Phosphorylation of Castor Bean RcSUS1

Ser(P)-11. These results indicate that RcSUS1 was stoichiometrically phosphorylated at Ser-11 in stage III–V COS in vivo, whereas RcSUS1 purified from stage V–VII COS had a phosphorylation stoichiometry of ~0.7 mol/mmol of p93 subunits. This was corroborated by anti-Ser(P)-11 immunoblotting, which demonstrated that relative Ser-11 phosphorylation of p93 was maximal during the early stages of COS development but progressively declined as the seed matured (Fig. 7D).

A radiometric SUS kinase assay was developed that employed the synthetic RcSUS1 dephosphopeptide and [γ-32P]ATP as substrates and a butyl Sepharose-enriched stage from stage V–VII developing COS endosperm as the kinase source. SUS kinase activity was detected that catalyzed Ca2+-dependent phosphorylation of the dephosphopeptide at Ser-11 (Fig. 7E). Similarly, a Ser(P)-11-specific ELISA demonstrated Ca2+-dependent rephosphorylation of purified RcSUS1 that had been in vitro dephosphorylated with λ-phosphatase (Fig. 7F).

RcSUS1 Activity, Protein Expression, and Ser-11 Phosphorylation Appear to Be Modulated by Sucrose Recently Translocated from Source Leaves—To assess the effect that photosynthesis supply to developing COS has on RcSUS1 activity, protein expression, and Ser-11 phosphorylation, stems containing intact pods of castor fruits were excised and incubated in the dark for up to 72 h. SUS activity and RcSUS1 (p93) protein abundance of stage VII COS both remained relatively constant for the first 48 h but then showed a significant decrease by 72 h (Fig. 7, G and H), which parallels the corresponding elimination of RcSUS1 transcripts (Fig. 2D). However, phosphorylation of p93 at Ser-11 steadily decreased over the depodding time course such that it was largely abolished by 72 h (Fig. 7F).

Microsome-associated RcSUS1 Is Hypophosphorylated Relative to Soluble RcSUS1—To determine whether any SUS is associated with endomembranes, microsomes were isolated from freshly collected stage V–VII developing COS. Although the majority of SUS activity and immunoreactive p93 polypeptides remained in the soluble fraction, 12 ± 1% of total SUS activity and 11 ± 1% (means ± S.E. of n = 3 biological replicates) of immunoreactive RcSUS1 (p93) were located in the microsomal fraction. Anti-Ser(P)-11 immunoblotting established that the relative phosphorylation stoichiometry of microsomal associated RcSUS1 was 45 ± 8% (mean ± S.E. of n = 3 biological replicates) that of soluble RcSUS1.

Depodding Triggers RcSUS1 Dephosphorylation but Does Not Alter Its Partitioning between the Soluble and Microsomal Membrane Fractions—The relationship between photosynthesis supply and partitioning of RcSUS1 between soluble and microsomal membrane fractions was assessed by incubating excised pods of stage V–VII developing COS for 48 h in the dark. In agreement with the results of Fig. 7I, depodding triggered an approximate 50% decline in the relative phosphorylation of RcSUS1 at Ser-11 in both the soluble and microsomal membrane fractions (Fig. 8A). However, the proportion of RcSUS1 associated with microsomal membranes was unchanged following this treatment (Fig. 8B).

RcSUS1 Kinetic Properties—Similar to SUS from other plant sources, the activity of RcSUS1 in the cleavage direction exhibited: (i) a broad pH/activity profile with a maximum between pH 6.5 and 7.0, whereas its pH optimum in the direction of sucrose synthesis was >9.0 (Fig. 9A); (ii) hyperbolic sucrose and UDP saturation kinetics [Km(sucrose) = 32 ± 2 mm; Km(UDP) = 84 ± 5 μM] (Fig. 9); (iii) no dependence for a metal cation co-factor, including Mg2+ or Mn2+; and (iv) potent inhibition by Cu2+ and Zn2+, with 1 mM CuSO4 or ZnCl2 exerting 93 and 99% inhibition, respectively. RcSUS1 activity at physiological pH is clearly poised in the direction of sucrose cleavage (forward/reverse activity ratio = 1.9 ± 0.03 and 10.2 ± 0.4 at pH 7.1 and 6.6, respectively). In vitro dephosphorylation of the purified enzyme with λ-phosphatase did not significantly alter its: (i) pH activity profile in the cleavage (assayed with either saturating or subsaturating sucrose) or synthesis directions (Fig. 9A) or (ii) sucrose or UDP saturation kinetics in the sucrose-cleaving direction (Fig. 9, B and C). RcSUS1 appears to exclusively utilize UDP as its nucleoside diphosphate co-substrate, because no cleavage activity was detected when UDP was substituted with ADP, GDP, or CDP.

There is clear evidence for 14-3-3 protein involvement in the post-translational control of several key enzymes of primary plant metabolism, particularly sucrose-phosphate synthase and nitrate reductase (1, 38). Proteomic surveys of 14–3–3 client proteins from developing Arabidopsis and barley seeds identi-
In Vivo Phosphorylation of Castor Bean RcSUS1

The castor SUS family comprises five genes that exhibited distinctive expression profiles with RcSUS1 being the dominant isoform of developing COS (Fig. 2, A–C) (40, 41). RcSUS1 belongs to the dicot SUS1 group and has high sequence identity with soybean root nodule (GmSUS1) and Arabidopsis anoxia-inducible (AtSUS1 and AtSUS4) orthologs (Table 2) (5, 21). LTQ-FT MS/MS analysis of the purified SUS established RcSUS1 as the foremost SUS isoform of developing COS (Fig. 5A). This corroborates a proteomic study that detected a single SUS isoform corresponding to RcSUS1 during COS maturation (9). The high activity of SUS relative to invertase in COS (Fig. 3) likely reflects the fact that sucrose conversion to hexose phosphates via SUS requires less ATP than conversion via invertase (Fig. 1). SUS has been frequently proposed as the favored route of sucrolysis in metabolically active, bulky organs such as COS, where ATP synthesis via oxidative phosphorylation is likely restricted because of hypoxic conditions (45).

Although typically classified as a soluble cytosolic enzyme, SUS has been localized to various extra- and intracellular compartments including the cell wall, plasma membrane, Golgi, mitochondrial matrix, nucleus, tonoplast, plastids, and cytoskeleton (19, 24–26, 46–50). However, imaging of transiently expressed fluorescent fusions in tobacco suspension cells, a well-established model for heterologous plant cell biology studies (51), demonstrated the cytosolic location of RcSUS1 (Fig. 6). A noteworthy kinetic feature of RcSUS1 was that its sucrose cleavage activity was unable to employ ADP, GDP, or CDP as an alternative nucleoside diphosphate substrate to UDP. This may be a unique property of oil seed SUS, because SUS isozymes of starch-storing tissues readily employ ADP as a co-substrate (3, 13).

Concentrations of sucrose (20 mM) and UDP (0.1 mM). The following compounds had no effect on phospho- or dephospho-RcSUS1 activity (±25% control velocity): coenzyme A, acetyl-CoA, malonyl-CoA, AMP, KNO₃, oxalate, and CaCl₂ (2 mM each); 6-phosphogluconate, ribose-5-phosphate, glucose-1-phosphate, fructose-1,6-biphosphate, dihydroxyacetone-phosphate, glyceraldehyde-3-phosphate, glyceraldehyde-3-phosphate, glycerol, 3-phosphoglycerate, 2-phosphoglycerate, 2-phosphoglycerol, PEP, pyruvate, α-ketoglutarate, citrate, fumarate, isocitrate, malate, succinate, oxaloacetate, alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, ATP, and NaPi (5 mM each). However, 10% (v/v) glycerol reduced V_max by >50% in the sucrose cleavage direction by functioning as a mixed competitive inhibitor with respect to sucrose (Fig. 9C).

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In Vivo Phosphorylation of Castor Bean RcSUS1

Developmental patterns of SUS activity and immunoreactive RcSUS1 (p93) polypeptides during COS maturation (Fig. 3) were similar to those reported for seed SUS orthologs from other species. Maximal SUS activity and/or protein expression typically occurs during mid- to late development when the greatest rates of seed storage end product deposition occur (4, 5, 7, 8, 10–12, 16, 23). By contrast, relative phosphorylation of RcSUS1 p93 subunits at Ser-11 peaked during the early stages of COS development and then progressively decreased as the seed matured (Fig. 7D). This contrasts with phosphorylation of the plant-type PEP carboxylase RcPPC3 at Ser-11, which was greatest in stage VII COS and then significantly declined by stage IX (36, 54). Stages V–VII represent the major phase of storage oil and protein accumulation in COS endosperm (53). At stage IX, where minimal phosphorylation of RcSUS1 was observed (Fig. 7D), the seed is almost mature, has lost vascular connection with the parent plant, and has initiated the desiccation phase (32). Similarly, a steady decline in relative Ser-11 phosphorylation, followed by a decrease in RcSUS1 protein abundance and activity, was caused by a depodding-induced elimination of photosynthate delivery to COS fruit clusters (Fig. 7, G–I). The results: (i) implicate a direct inverse relationship between RcSUS1 expression and Ser-11 phosphorylation and the supply of sucrose from source leaves to the nonphotosynthetic COS (ii) are reminiscent of the depodding-induced dephosphorylation of COS plant-type PEP carboxylase (RcPPC3) (36, 54), but (iii) contrast with the depodding-enhanced multisite in vivo phosphorylation of bacterial-type PEP carboxylase (RcPPC4) at Ser-425 and Ser-451 (37, 55). The ability of plant cells to sense sugars plays a crucial role in C-partitioning and allocation between source and sink tissues. These processes are modulated as a consequence of the plant’s sugar status, and sugar signals function both at the transcriptional, translational, and post-translational levels in tight coordination with light and other environmental stimuli (1, 2). A key area for future studies will be to establish signaling pathways that link sucrose supply with the differential expression and in vivo phosphorylation of key metabolic enzymes such as SUS and PEP carboxylase that are involved in the control of photosynthate partitioning in heterotrophic sink tissues, including developing seeds and legume root nodules.

Previous evidence for in vivo phosphorylation of seed SUS was provided by: (i) Haigler et al. (30), who demonstrated the covalent incorporation of 32P into unspecified site(s) of SUS following incubation of developing cotton embryos with 32P, and Duncan et al. (23), who employed immunoblotting using a phosho-site specific antibody to demonstrate phosphorylation of the SUS isozyme SUS-SH1 at Ser-10 during maize kernel maturation. However, no information on the developmental profile, stoichiometry, or impact of phosphorylation on SUS functional properties during seed filling is currently available. The overall pattern of in vivo seryl-phosphorylation of RcSUS1 in COS, as well as the phosphorylation motif flanking its Ser-11 residue (Fig. 5A), are distinct from those of COS plant- and bacterial-type PEP carboxylases (36, 37, 39, 55). These differences implicate novel kinase-phosphatase pairings in controlling the phosphorylation status of RcSUS1, and plant- and bacterial-type PEP carboxylases in developing COS. In particular, the Ca2+-dependent RcSUS1 kinase activity (Fig. 7, E and F) contrasts with the Ca2+-independent protein kinase that in vivo phosphorylates plant type PEP carboxylase (RcPPC3) at Ser-11 in developing COS (54) but is analogous to CDPKs that phosphorylate SUS orthologs from several other plant species (17, 21, 27, 56). It is notable that the CDPK, which phosphorylates COS bacterial-type PEP carboxylase at Ser-451 in vivo, was unable to phosphorylate RcSUS1 or its corresponding synthetic dephosphopeptide (39). Future studies are needed to identify genes encoding castor CDPK isozymes that phosphorylate RcSUS1 in vivo at Ser-11 versus bacterial-type PEP carboxylase at Ser-451 in developing COS.

Studies of SUS isozymes from expanding maize leaves, pear and tomato fruits, and mung bean seedlings indicated that phosphorylation at their conserved N-terminal seryl residue activates the enzyme by increasing its affinity for sucrose and UDP (17, 27–29, 31). However, the activity of soybean nodule SUS (to which RcSUS1 is most closely related; Table 2) was unaffected by phosphorylation or by phosphomimetic mutagenesis of its target phospho-site (S11D) (21). Similarly, we were unable to detect any obvious impact of (de)phosphorylation on the kinetic properties of RcSUS1 (Fig. 9). Eukaryotic regulatory proteins known as 14-3-3s bind to phosphorylated serine and threonine residues of intracellular target proteins to control target protein function (38, 43, 44, 57). There is clear evidence for 14-3-3 protein involvement in the control of several key enzymes of primary plant metabolism, particularly sucrose-phosphate synthase and nitrate reductase (1, 38). Proteomic surveys of 14-3-3 client proteins from developing Arabidopsis and barley seeds identified SUS as a putative 14-3-3 target, even though SUS lacks a canonical 14-3-3 binding motif ((R/K)XX(pS/pT)XP or (R/K)XXX(pS/pT)P) (43, 44). Moreover, preincubation with an excess of a recombinant 14-3-3 protein was reported to cause an approximate 50% inhibition of the sucrose synthesis activity of SUS from developing barley endosperm clarified extracts that had been preincubated with ATP (41). However, incubation of RcSUS1 with a yeast 14-3-3 protein (BMH2) had no detectable influence on SUS activity in either direction. BMH2 has been used to identify and characterize several plant 14-3-3 binding phosphoproteins, including nitrate reductase (38, 57).

The lack of an effect of Ser-11 phosphorylation or 14-3-3 proteins on RcSUS1 activity indicates that phosphorylation has an alternative role. For example, Ser-11 phosphorylation of soybean SUS reduced its hydrophobicity and may promote redistribution of the enzyme from microsomal membranes into the soluble fraction of root nodules (18, 21). Analogous results were obtained with SUS orthologs from the elongating zone of maize leaves (17, 20, 23). Similarly, we demonstrated that ~10% of total SUS protein and activity was associated with the COS microsomal fraction and that microsome-associated SUS was hypophosphorylated relative to soluble SUS. However, in vivo dephosphorylation of RcSUS1 caused by a depodding-induced elimination of photosynthate supply to developing COS had no impact on its partitioning between soluble and microsomal membrane fractions (Fig. 8). Thus, hypophosphorylation of RcSUS1 appears to be a consequence rather than a cause of its microsomal membrane association. Furthermore: (i) relative
Ser-11 phosphorylation of RcSUS1 decreased over the course of COS development (Fig. 7D), followed by elimination of RcSUS polypeptides and activity in fully mature COS (Fig. 3); (ii) depodding of castor fruits also prompted a steady decline in Ser-11 phosphorylation, which was followed by a pronounced reduction in levels of RcSUS1 protein and activity (Fig. 7, G–I); (iii) Ser-11 phosphorylation of soybean root nodule SUS was hypothesized to mitigate its proteolytic turnover in vivo (18); and (iv) seeds of a spk rice mutant lacking a CDPK isoform that phosphorylates SUS in vitro exhibited reduced SUS activity and polypeptide levels while accumulating sucrose at the expense of starch (56). Thus, it seems plausible that in vivo dephosphorylation by an as yet unspecified protein phosphatase enhances the proteolytic susceptibility of RcSUS1. If so, high stoichiometric phosphorylation at Ser-11 would be critical to maintaining the abundant levels of RcSUS1 protein and activity that characterize intact stage III–VII developing COS.

In conclusion, respiration, storage end product biosynthesis, and carbon-nitrogen interactions in developing seeds are entirely dependent upon the translocation of photosynthate from photosynthetic tissues. SUS plays a key role in this process by cleaving sucrose to conserve ATP while supplying hexose phosphates and sugar nucleotides for downstream metabolic and biosynthetic pathways. RcSUS1, the dominant sucrolytic enzyme of developing COS, strictly uses UDP for sucrose cleavage, which may be a unique feature of sucrolysis in a nonstarch accumulating oil seed. Although RcSUS1 is subject to high stoichiometric and dynamic Ser-11 phosphorylation in vivo, phosphorylation did not affect its activity or kinetic properties or partitioning between the soluble cytosol and microsomal membranes. RcSUS1 phosphorylation appears to closely correlate with its resistance to proteolytic turnover. It will therefore be of considerable interest to directly establish the impact of Ser-11 phosphorylation on RcSUS1 susceptibility to intracellular proteases.

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