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Seryl-phosphorylation of soybean nodule sucrose synthase (nodulin-100) by a Ca^{2+}-dependent protein kinase

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Abstract Sucrose synthase (SS; EC 2.4.1.13) was radiolabeled in situ by incubating detached soybean nodules with ^{32}Pi. Phosphoamino acid analysis indicated that SS was phosphorylated on a serine residue(s). In-vitro phosphorylation of purified nodule SS by desalted nodule extracts was Ca^{2+}-dependent. This SS-kinase was partially purified (~2200-fold) from nodules harvested from illuminated plants. The molecular mass of the SS-kinase was about 55 000 on a Superdex 75 size-exclusion column or in a denaturing autophosphorylation gel. With either purified nodule SS or Syntide 2 as substrate, exogenous calmodulin and phosphatidylserine showed little or no effect on the in-vitro activity of this partially purified protein kinase. However, its activity was inhibited by W-7. The purified nodule SS-kinase (or CDPK) phosphorylated nodule PEP carboxylase (PEPC; EC 4.1.1.31) in the presence of Ca^{2+}. In contrast, a partially purified nodule PEPC-kinase preparation was incapable of phosphorylating nodule SS. Unlike nodule PEPC [Zhang et al. (1995) *Plant Physiol.* 108, 1561–1568], the phosphorylation state of SS is not likely modulated in planta by photosynthate supply from the shoots.

Key words: Nodulin-100; Protein kinase; Protein phosphorylation; Root nodule; Soybean (*Glycine max*); Sucrose synthase

1. Introduction

Sucrose synthase (SS) is an important sucrose-cleaving enzyme in a variety of plant 'sink' tissues (e.g. tubers, developing leaves and grains, root nodules; [1–6] and refs. therein) by virtue of its reversible conversion of sucrose and UDP into UDP-Glc and Fru. In the legume *Vicia faba*, the *SS* gene is expressed predominantly in the N_2-fixing root nodules as compared to the uninfected roots, mature leaves and seeds, and hypocotyl, stem and flower tissues [6]. Similarly, Thummler and Verma [5] established that one of the major nodule-enhanced proteins (nodulin-100) in soybean is the ~90-kDa subunit of tetrameric SS. The levels of both the nodulin-100 protein and transcript are higher in nodules than in other soybean tissues [5,7]. Physiologically, it is believed that SS plays a critical role in cleaving sucrose translocated to the nodules from the shoots in support of N_2 fixation [4–9]. The resulting hexoses (and Glc 1-*P*) enter glycolysis in the host-cell cytosol to form PEP, the carboxylation substrate for cytosolic PEPC. The PEPC-derived C_4 acids (e.g. malate, succinate) serve as respiratory substrates for the N_2-fixing bacteroids and also provide carbon skeletons for the assimilation of the resulting NH_4^+.

Control of SS activity in plants is thought to primarily involve transcriptional regulation of the steady-state level of enzyme protein [1–3,10,11]. In addition to developmental and tissue/cell-specific control, the expression of the nuclear *SS* genes is known to be modulated by various stimuli, including tissue sugar-status and anaerobiosis [[11,12] and refs. therein]. In marked contrast, regulatory mechanisms for the control of SS activity per se are not well understood [4,5]. Encouraged by the observation that the SS protein was radiolabeled in situ when cultured maize suspension cells were incubated with [γ-^{32}P]ATP [12], we investigated the potential for control of SS activity in the legume nodule by protein phosphorylation. Here we report that SS (nodulin-100) in soybean root nodules is phosphorylated in situ at a serine residue(s). The nodule SS-kinase was partially purified by an FPLC-based protocol and shown to be a soluble, ~55-kDa CDPK. Similarly, Huber et al. [13] very recently reported that the SS-2 isoform in the elongation zone of maize leaves was phosphorylated on a conserved serine residue (Ser^{15}) by a ~65-kDa CDPK. This protein kinase was identified and partially purified using a synthetic peptide as substrate.

2. Materials and methods

2.1. Plant material

Soybean (*Glycine max* [L.] Merr. cv Dunbar) plants were inoculated, grown in a local greenhouse, and pretreated by high-light exposure or stem girdling as described previously [14].

2.2. Purification of soybean-nodule SS and PEPC

SS was purified to electrophoretic homogeneity by $(NH_4)_2SO_4$ fractionation (30–45% saturation) of a clarified nodule extract and sequential FPLC on phenyl Sepharose, Mono Q, and phenyl boronate agarose (Amicon; see [4]). The final preparation had a specific activity of 9.5 μmol UDP-Glc formed min^{-1} mg^{-1} protein and was free of detectable PEPC activity and its ~110-kDa polypeptide. The purification procedure will be described in detail elsewhere.

Nodule PEPC from stem-girdled plants was purified to electrophoretic homogeneity as described in [14].

2.3. In-situ ^{32}P-labeling, immunoprecipitation, and phosphoamino acid analysis of nodule SS

See Zhang et al. [14] for experimental protocols. The anti-maize kernel SS antibodies used for indirect immunoprecipitation were kindly provided by Dr. Karen E. Koch, University of Florida [see [11]].

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Abbreviations: CDPK, Ca^{2+}-dependent protein kinase; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; FPLC, fast-protein liquid chromatography; MC-LR, microcystin-LR; PEG, polyethylene glycol; PEP, phospho*enol*pyruvate; PEPC, PEP carboxylase; PEPC-K, PEPC-kinase; PMSF, phenylmethylsulfonyl fluoride; SS, sucrose synthase; SS-K, SS-kinase; TLE, thin-layer electrophoresis; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide

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2.4. Partial purification of nodule SS-kinase and PEPC-kinase

Frozen nodules (50 g) from high-light illuminated plants were ground at 0–4°C with a mortar and pestle in 200 ml of extraction buffer containing 100 mM Mops-KOH, pH 7.3, 10% (v/v) glycerol, 15 mM 2-mercaptoethanol, 5 mM EGTA, 0.5 g insoluble polyvinylpyrrolidone, 10 μg/ml chymostatin, 5 μg/ml E-64, 2.5 μg/ml 3,4-dichloroisocoumarin, 1 μg/ml leupeptin and pepstatin A, and 1 mM fresh PMSF. The homogenate was passed through six layers of cheesecloth and clarified by centrifugation at 38 000×*g* for 30 min. PEG-8000 was added to the supernatant fraction to 5% (w/v), followed by stirring for 15 min at 0–4°C. After centrifugation as above, additional PEG was added to the supernatant fluid to a final concentration of 26%. This suspension was stirred for another 20 min, and the precipitate was collected by centrifugation at 30 000×*g* for 15 min. The pellet was resuspended in buffer A (20 mM Mops-KOH, pH 7.3, 1 mM EGTA, 50 mM NaCl, 5 mM 2-mercaptoethanol) containing leupeptin, E-64, and pepstatin A, and clarified by centrifugation. This sample was loaded onto a DE-52 (Whatman) column (2.5×10 cm) equilibrated with buffer A. The column was washed with buffer A until A_{280} approached baseline, and then with a 50–500 mM linear gradient of NaCl in buffer A. The peak SS-kinase activity fractions were pooled and thoroughly dialyzed against buffer B (20 mM Tris-HCl, pH 7.3, 1 mM $CaCl_2$). This sample was loaded onto a phenyl Sepharose CL-4B (Pharmacia) column (1.5×15 cm) equilibrated with buffer B. The column was washed with 200 ml buffer B, and then kinase activity was eluted with 20 mM Tris-HCl, pH 8.0, 5 mM EGTA. Leupeptin and E-64 were re-added to the pooled SS-kinase fractions. This kinase sample was concentrated to 200 μl with a Centricon 10 centrifugal concentrator and loaded onto a prepacked Superdex 75 HR 10/30 (Pharmacia) column equilibrated with 20 mM Tris-HCl, pH 7.3, 0.1 M NaCl, 0.5 mM EGTA, and 5 mM 2-mercaptoethanol. The kinase was eluted with this same buffer. The size-exclusion column was calibrated with various protein standards (66–12.4-kDa range) in order to estimate the native molecular mass of SS-kinase. The pooled kinase fractions from the Superdex column were loaded directly onto a prepacked Mono Q HR 5/5 (Pharmacia) column equilibrated with buffer C (20 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 5 mM 2-mercaptoethanol, 0.1 M NaCl). SS-kinase activity was eluted with a linear gradient of 0.1–0.5 M NaCl in buffer C. The kinase fractions were combined, concentrated with a Centricon 30, and stored at −70°C. All of the above chromatographic steps were performed by FPLC at 0–4°C.

Nodule PEPC-kinase was isolated as described in detail elsewhere [15].

2.5. Enzyme assays

The activity of SS in the sucrose-cleavage direction was assayed spectrophotometrically at 340 nm and 30°C by coupling to excess UDP-Glc dehydrogenase (Boehringer Mannheim) as described in [2,4], in a final volume of 1 ml.

The activity of SS-kinase was assayed by ^{32}P-incorporation from [γ-^{32}P]ATP into purified nodule SS or PEPC, or Syntide 2 (Calbiochem), as indicated. In a 40-μl reaction mixture, ~12 μg purified SS or PEPC, or 20 μM Syntide 2, was incubated with the appropriate amount of kinase, 50 mM HEPES-KOH, pH 7.5, 5 mM $MgCl_2$, 0.1 μM MC-LR, and 5 μCi [γ-^{32}P]ATP (3 Ci/mmol) for 10 min at 30°C in the presence of Ca^{2+} or EGTA as indicated. When SS or PEPC was used as substrate, the polypeptides in the reaction mixture were separated by SDS-PAGE, and the ^{32}P incorporated into the ~90- or ~110-kDa SS or PEPC subunits, respectively, was detected/quantified by phosphorimaging. When Syntide 2 was used as substrate, a 25-μl aliquot of the reaction mixture was transferred into a Pierce phosphocellulose separation unit (SpinZyme format) and further processed according to the manufacturer's instructions. The ^{32}P-radioactivity incorporated into Syntide 2 was then measured by Cerenkov counting in a liquid scintillation spectrometer.

The in-gel assay of SS-kinase autophosphorylation activity was carried out as described elsewhere [15,16], in the presence of Ca^{2+} or EGTA as indicated.

PEPC activity was determined as described [14]. The in-vitro phosphorylation of purified nodule PEPC or SS by partially purified nodule PEPC-kinase was performed as described [15], in the presence of Ca^{2+} or EGTA as indicated.

3. Results and discussion

3.1. Soybean nodule SS is phosphorylated in situ on a serine residue(s)

In order to examine whether soybean nodule SS is phosphorylated in vivo, detached nodules from illuminated or stem-girdled plants were incubated with ^{32}Pi for 2 h. As indicated in Fig. 1, the ~90-kDa SS subunit was radiolabeled in nodules from both sets of plants on a serine residue(s). Notably, phosphorimaging analysis indicated that there was no significant difference in ^{32}P incorporation into the SS polypeptide from the control nodules harvested from illuminated plants and the sucrose-depleted nodules harvested from stem-girdled plants. To our knowledge, this is the first evidence to document that legume nodulin-100 is phosphorylated in situ on a serine residue(s), similar to the very recent report of SS phosphorylation in the elongation zone of maize leaves [13].

3.2. Characterization of nodule SS-kinase

In order to determine the Ca^{2+} dependence of SS phosphorylation in vitro, purified nodule SS was incubated with a desalted nodule soluble extract prepared from illuminated soybean plants and [γ-^{32}P]ATP/Mg^{2+}, ± $CaCl_2$ or EGTA. As shown in Fig. 2A, nodulin-100 was phosphorylated in the presence of exogenous Ca^{2+} but not in its absence, suggesting that a CDPK may be involved in the phosphorylation of nodule SS. During the final stages of this study, Huber et al. [13] likewise reported that the SS-2 isoform in the elongation zone of maize leaves is phosphorylated in vitro in a Ca^{2+}-dependent manner.

The related in-vitro results in Fig. 2B suggest that there is no significant difference in total SS-kinase activity between nodules from illuminated and stem-girdled plants. This observation is consistent with the results from the in-situ ^{32}P labeling of nodulin-100 (see Fig. 1A). These findings represent a striking contrast to PEPC-kinase activity and PEPC phosphorylation in soybean nodules. In this latter case, both kinase activity and the phosphorylation state of the target protein are up-regulated in illuminated plants as compared to stem-girdled or extensively darkened plants [14,15].

To further characterize this soluble, Ca^{2+}-dependent protein kinase from soybean nodules, an FPLC-based purification protocol was established on the basis of published procedures for the isolation of plant CDPK [17–19]. During the entire purification process, purified nodule SS was used as substrate for detection of protein kinase activity. By the protocol outlined in Section 2.4, about a 2200-fold purification was achieved. On a Superdex 75 size-exclusion column, SS-kinase activity eluted at around 55 kDa. The data in Fig. 3A indicate that phosphorylation of SS by this partially purified protein kinase is Ca^{2+}-dependent. In addition, the ~55-kDa polypeptide that is also phosphorylated intensely in the presence of Ca^{2+} is likely the autophosphorylated SS-kinase monomer. This possibility was explored directly by electrophoresing the partially purified kinase sample in an SDS-gel, followed by denaturation/renaturation and assay in situ in the presence of [γ-^{32}P]ATP/Mg^{2+}, ± Ca^{2+} or EGTA. Indeed, a ~55-kDa renaturable polypeptide was detected by this in-gel autophosphorylation assay in the presence of Ca^{2+}, but not in its absence (Fig. 3B).

With nodule SS as substrate, both exogenous calmodulin (1 μM) and phosphatidylserine (25 μg/ml) showed little or no

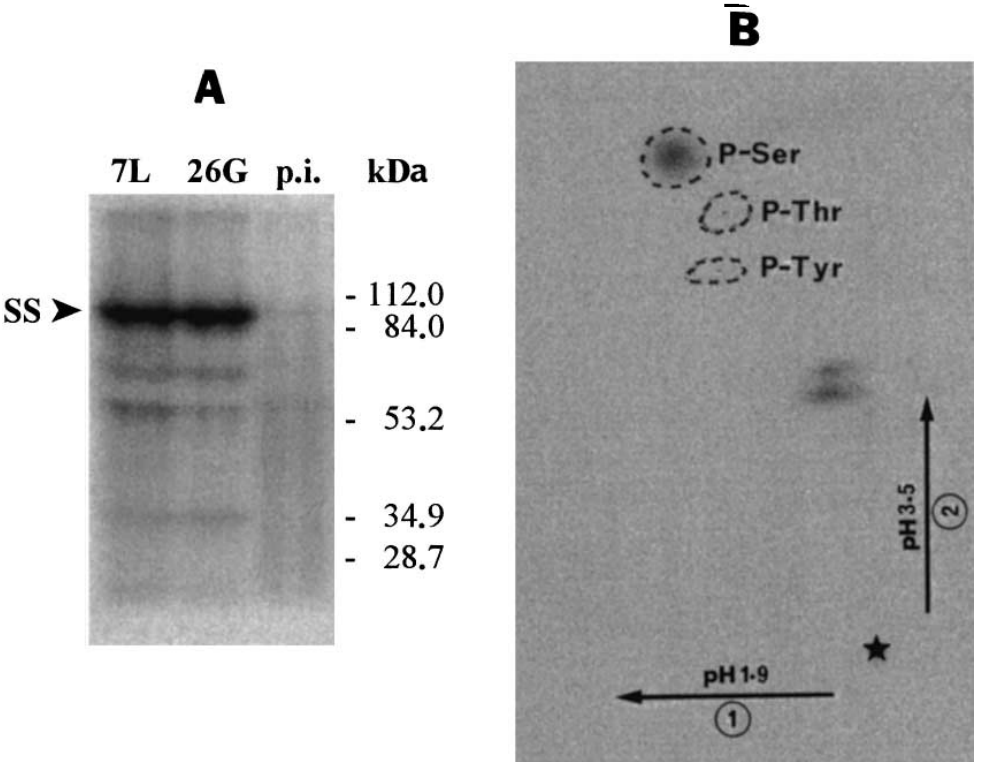

Fig. 1. In-situ ^{32}P-labeling (A) and phosphoamino acid analysis (B) of soybean nodule SS. Detached nodules from illuminated (7 h (7L)) and stem-girdled (26 h (26G)) plants were incubated with 250 μCi ^{32}Pi for 2 h, and soluble protein extracts were prepared and incubated with anti-maize kernel SS antibodies [see [11]] or pre-immune serum (p.i.). The immune complexes were recovered using protein A-Sepharose beads, thoroughly washed, and the ^{32}P-labeled polypeptides were resolved by SDS-PAGE and visualized by phosphorimaging (A). Alternatively, following electrophoresis, proteins were transferred to an Immobilon-P membrane. The ~90-kDa, ^{32}P-labeled SS subunit-band was then excised and acid hydrolyzed. Radiolabeled amino acids were resolved by 2-dimensional TLE and detected by phosphorimaging (B). The star in (B) is the origin for TLE.

effect on the in-vitro activity of this nodule kinase in the presence of Ca^{2+}. However, the inclusion of 150 μM W-7 in the assay system inhibited Ca^{2+}-dependent kinase activity by about 50%. Syntide 2, a routine peptide substrate for plant CDPK [20] and Ca^{2+}/calmodulin-dependent protein kinases, is also a good substrate for the partially purified protein kinase. When Syntide 2 was used as substrate, the in-vitro activity of this nodule kinase was again not responsive to either calmodulin or phosphatidylserine, but strongly inhibited (76%) by 150 μM W-7. Collectively, this partially purified nodule SS-kinase preparation has biochemical characteristics typical of a plant CDPK [17–20].

SS and PEPC represent the only known metabolic enzymes subject to seryl-phosphorylation in legume nodules (Fig. 1) [14]. Given this common feature, the 'cross-phosphorylation' between nodule SS-kinase, SS, PEPC-kinase, and PEPC was examined in vitro. The partially purified, Ca^{2+}-dependent SS-kinase was able to phosphorylate purified nodule PEPC in the presence of Ca^{2+} (Fig. 4). In contrast, the partially purified, Ca^{2+}-independent PEPC-kinase [15] was unable to phosphorylate purified nodule SS in the presence or absence of Ca^{2+} (Fig. 4). This provides additional evidence for our view [15,21] that PEPC-kinase is a highly specific protein-Ser/Thr kinase dedicated to plant PEPC, especially when one considers that both tetrameric PEPC and SS have an homologous phosphorylation motif (basic–x–x–*Ser*–hydrophobic) near the N-terminus [13,21,22]. For example, PEPC from developing soybean seeds and alfalfa root nodules has a phosphorylation domain of –K–M–A–S^{11}–I– and –K–M–A–S^{10}–I–, respectively [see [22]]. Similarly, all three published legume SS sequences have a related phosphorylation motif of –R–V–H–S^{11}–L– [6,13,23,24]. Although, as expected [21,22], nodule PEPC was an in-vitro substrate for the nodule CDPK preparation (Fig. 4), there is no evidence that a plant CDPK is involved directly in the regulatory phosphorylation of PEPC in vivo [21,22].

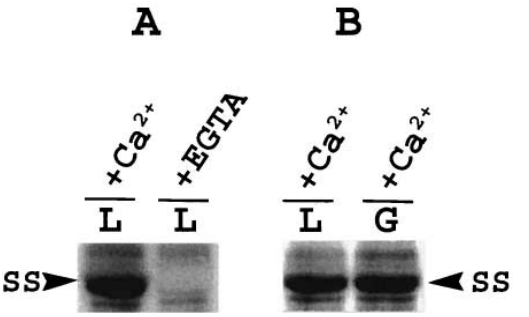

Fig. 2. A soluble, Ca^{2+}-dependent protein kinase is involved in the phosphorylation of nodule SS (A), and this kinase activity is not affected by stem-girdling pretreatment of soybean plants (B). Desalted nodule crude extracts were prepared from illuminated (L) and stem-girdled (G) plants, incubated with 12 μg purified soybean SS, [γ-^{32}P]ATP/Mg^{2+}, 0.1 μM MC-LR, ±0.25 mM Ca^{2+} or 0.5 mM EGTA, at 30°C in a 40-μl assay mixture as described in Section 2.5. Following a 10-min incubation and subsequent denaturation, phosphorylated polypeptides were resolved by SDS-PAGE and detected by phosphorimaging.

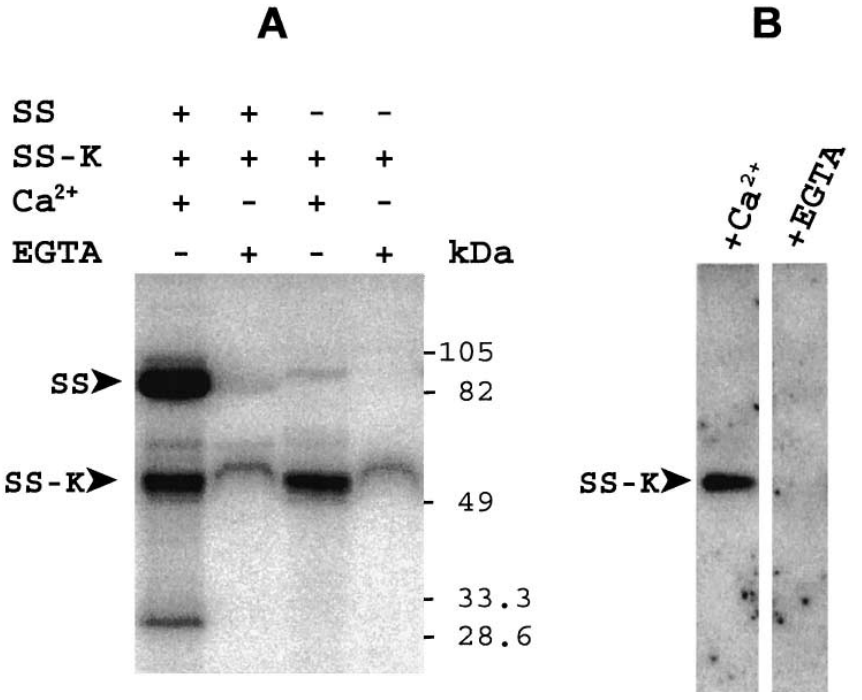

Fig. 3. In-vitro phosphorylation of nodule SS by the partially purified SS-kinase (A), and detection of autophosphorylation by this purified nodule CDPK by an in-gel assay (B). Phosphorylation of purified SS was performed as described in Section 2.5. and Fig. 2. For in-gel assay of autophosphorylation activity, the purified nodule kinase sample was electrophoresed in a 10% SDS mini-gel, followed by in-situ denaturation/renaturation and assay, ±0.25 mM Ca^{2+} or 1 mM EGTA. The autophosphorylated polypeptide was detected by phosphorimaging.

3.3. Conclusion

To our knowledge, this study provides the first evidence that soybean nodulin-100 is phosphorylated in situ on a serine residue(s) by a soluble CDPK. With these findings, SS now represents only the third specific, plant-encoded protein known to undergo phosphorylation in the legume nodule. Previously, nodulin-26, an ion- or water-channel protein in the symbiosome membrane, was shown to be phosphorylated at Ser^{262} by a membrane-associated CDPK in soybean nodules [18,25]. More recently, we reported that cytosolic PEPC, which plays essential roles in the synthesis of C_4 acids for the N_2-fixing bacteroids and in the provision of carbon skeletons for the assimilation of the resulting NH_4^+, was phosphorylated in soybean nodules at a serine residue (likely Ser^{11}) by a Ca^{2+}-independent protein kinase (see Fig. 4) [14,15]. Thus, the present study provides additional evidence for our view that protein phosphorylation is likely a potentially important mechanism for the control of carbon metabolism and transport in the legume nodule. To date, little is known about the regulation of the phosphorylation state of nodulin-26 in situ. In contrast, the phosphorylation of nodule PEPC is controlled largely by PEPC-kinase activity which, in turn, is regulated by photosynthate recently transported from the shoots [14,15]. It appears that the mechanism(s) governing SS phosphorylation in soybean nodules is likely to differ from that controlling PEPC phosphorylation because neither the phosphorylation state of SS nor the total activity of its CDPK is responsive to a decreased supply of photosynthate to the nodules from the shoots (Fig. 1A and 2B). Another critical but as yet unanswered question with respect to the phosphorylation of SS in the legume nodule (this study), developing leaves [13], and *perhaps* in other plant 'sink' tissues is whether this covalent modification has any significant impact in planta on the enzyme's properties or its possible partitioning between the cytosol and plasma membrane [26,27].

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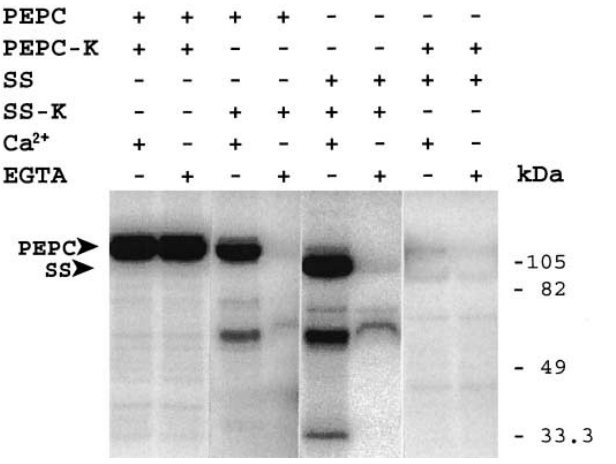

Fig. 4. Ability of the partially purified nodule SS-kinase to phosphorylate nodule PEPC, and inability of partially purified nodule PEPC-kinase to phosphorylate nodule SS. Purified nodule PEPC or SS (12 μg) was incubated individually with the partially purified SS-kinase or PEPC-kinase, 5 μCi [γ-^{32}P]ATP/Mg^{2+}, 0.1 μM MC-LR, ±0.25 mM Ca^{2+} or 0.5 mM EGTA, as indicated above the gel tracks.

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