CALCIUM-REGULATED PHOSPHORYLATION OF SOYBEAN SERINE ACETYLTRANSFERASE IN RESPONSE TO OXIDATIVE STRESS*

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Running title: Regulation of serine acetyltransferase by phosphorylation

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Glycine max serine acetyltransferase 2;1 (GmSerat2;1) is a member of a family of enzymes that catalyze the first reaction in the biosynthesis of cysteine from serine. It was identified by interaction cloning as a protein that binds to calcium-dependent protein kinase. In vitro phosphorylation assays showed that GmSerat2;1, but not GmSerat2;1 mutants (S378A or S378D), were phosphorylated by soybean calcium-dependent protein kinase isoforms. Recombinant GmSerat2;1 was also phosphorylated by soybean cell extract in a Ca2+-dependent manner. Phosphorylation of recombinant GmSerat2;1 had no effect on its catalytic activity, but rendered the enzyme insensitive to the feedback inhibition by cysteine. In transient expression analyses, fluorescently tagged GmSerat2;1 localized in the cytoplasm and with plastids. Phosphorylation state-specific antibodies showed that an increase in GmSerat2;1 phosphorylation occurred in vivo within 5 minutes of treatment of soybean cells with 0.5 mM hydrogen peroxide, whereas GmSerat2;1 protein synthesis was not significantly induced until one hour after oxidant challenge. Internal Ca2+ was required in the induction of both GmSerat2;1 phosphorylation and synthesis. Treatment of cells with calcium antagonists showed that externally derived Ca2+ was important for retaining GmSerat2;1 at a basal level of phosphorylation, but was not necessary for its hydrogen peroxide-induced synthesis. Protein phosphatase type 1, but not type 2A or alkaline phosphatase, dephosphorylated native GmSerat2;1 in vitro. These results support the hypothesis that GmSerat2;1 is regulated by calcium-dependent protein kinase phosphorylation in vivo and suggest that increased GmSerat2;1 synthesis and phosphorylation in response to active oxygen species could play a role in anti-oxidative stress response.

In plants and microorganisms, cysteine is the point of entry for inorganic sulfur into organic compounds. Cysteine synthesis proceeds via two steps:

1) L-serine + acetyl-CoA \(\rightarrow\) O-acetylserylserine + CoA
2) O-acetylserylserine + S2- \(\rightarrow\) L-cysteine + acetate.

In the first step, serine acetyltransferase (SAT\(^3\), EC 2.3.1.30) transfers an acetyl group from acetyl-CoA to serine to form the intermediate product, O-acetylserine (OAS). In the next reaction, catalyzed by O-acetylserylserine(thiol)-lyase (OAS-TL, EC 4.2.99.8), sulfide reacts with OAS, forming cysteine.

In plants, SAT and OAS-TL are found in multiple subcellular compartments, and the activity of SAT in these compartments is 4- to 300- fold less than that of OAS-TL (1-3). The availability of OAS has been found to be limiting for cysteine biosynthesis (4,5), and SAT is in a position to regulate not only cysteine synthesis, but also sulfur homeostasis for the whole plant (6). SAT activity is thought to be regulated by formation of a complex with OAS-TL(3,7) and some forms of plant SATs are feed-back inhibited by cysteine. In Arabidopsis, two of the three cytosolic SATs are inhibited by cysteine, while the plastidic and mitochondrial SATs are not (8,9). The cysteine-sensitive SAT activity of pea is in the...
plastid rather than in the cytosol (2). The variation between species in the sensitivity of plastidic and cytosolic SATs to cysteine suggests variation in the regulation of cysteine synthesis from species to species and compartment to compartment. Species differences in other parts of the sulfur assimilation pathway (10) further emphasize variation in sulfur metabolism and its regulation in plants.

Glutathione (γ-Glu-Cys-Gly), or homoglutathione (γ-Glu-Cys-βAla) in legumes including soybean, protects plants from oxidative damage that arises from various stress conditions. Considerable increases in the pool size of glutathione have been reported in plants exposed to various abiotic and biotic stresses such as chilling (11), drought (12), herbicide safeners (13), and pathogen attack (14). Increased glutathione concentrations were observed when cysteine content was increased artificially by supplying sulfate (15), fumigating with H₂S (16,17), direct feeding of cysteine (18,19), or overexpressing SAT (20), suggesting cysteine availability plays an important role in determining glutathione content. Recent evidence shows that in addition to increasing glutathione, stresses such as heat shock, chilling, hypoosmolarity, and exogenous application of H₂O₂ trigger increases in reactive oxygen species and cytosolic calcium in plant cells and seedlings (21-26), and that calcium is necessary for the stress response.

In this paper we show that a serine acetyltransferase from soybean (GmSerat2;1) is a substrate of CDPK. CDPKs (calcium-dependent protein kinases) function as both Ca²⁺ sensors and effectors (27). Phosphorylation of GmSerat2;1 at a specific serine residue in the carboxyl terminus renders the enzyme insensitive to feedback inhibition by cysteine. Using phosphorylation state-specific antibodies, we show that GmSerat2;1 is phosphorylated in soybean cells. Furthermore, treatment of cells with H₂O₂ induces both phosphorylation and protein synthesis of GmSerat2;1, and internally derived calcium and kinase activity are involved in the induction. Based on these results we propose that H₂O₂-induced increase in the activity of GmSerat2;1 contributes to the oxidative stress response in soybean.

**EXPERIMENTAL PROCEDURES**

**Plant Material** - Soybean cell suspension cultures (Glycine max L.) were maintained as described previously (28). Cells were subcultured every 7 days, and all treatments were done using cells 3 days after transfer to fresh medium. Inhibitors and H₂O₂ were added to the indicated concentrations. Following incubation for the indicated times, cells were collected on Miracloth (EMD Biosciences, San Diego, CA), drained of culture medium, and washed with 20 mM Tris, pH 8.5, 0.4 M sorbitol, 10 mM MgCl₂, 1 mM NaF, 1 mM Na₃VO₄ and 1 mM Na₂MO₄.

**DNA Sequencing** - Sequences of all constructs were verified by DNA sequencing performed by the University of Florida DNA Sequencing Core Facility. The sequence for GmSerat2;1AN (IP3) has been deposited in the GenBank Database under the accession number AY422685.

**Interaction Cloning** - Procedures for interaction cloning (identification of proteins expressed from a cDNA library that bind to a labeled probe) were adapted from Blanar and Rutter (29) and Stone et al. (30). A soybean cDNA library made in the UniZap™ XR vector, which allows expression of cDNA inserts as fusion proteins with β-galactosidase, was the gift of R. Tenhaken and C. Lamb. Plating of phage and expression and transfer of proteins to nitrocellulose membranes were performed using a picoBlue™ Immunoscreening Kit (Stratagene, La Jolla, CA). Nitrocellulose membranes were washed in 20 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 three times at 25 °C and incubated in 25 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM DTT, 5 mM MgCl₂, and 5% (w/v) nonfat dry milk for 1 – 2 h at 4 °C. Probes were purified recombinant soybean CDPKα, β, and γ labeled with ³²P by autophosphorylation in 100 µl phosphorylation buffer (31) for 15 min at 25 °C. After autophosphorylation and removal of unincorporated [γ-³²P] ATP by gel filtration, probes were equilibrated with binding buffer (25 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 1.2 mM CaCl₂, 2 mM DTT, and 50 mM NaCl), and the concentration adjusted to 2 – 5 x 10⁶ cpm/ml. Hybridization was performed at 4 °C overnight with continuous shaking in binding buffer supplemented with 1% nonfat dry milk.
Membranes were washed three times for 10 min each at 25 °C in the same buffer, air dried, and exposed to X-ray film.

**Complementation Assay** – *Escherichia coli* strain JM39 (F+, *cysE51*, *recA56*), which lacks serine acetyltransferase activity and requires L-cystine for growth (32), was transformed with putative SAT clones and tested for complementation by growth on medium lacking L-cystine.

**Plasmid Construction and Site-directed Mutagenesis** – To make a construct encoding maltose binding protein (MBP) fused to the N-terminus of GmSerat2;1, the open reading frame of clone IP3, which encoded interacting protein 3, was PCR-amplified and cloned into pMal-cRI (New England BioLabs). The 5’ primer was 5'-GCGGATCCCATATGGCCACTTGTGTT (BamH1, NdeI, underlined) and 3’ primer was 5'-GCGGATCCCTATATAACATAATCAGACCATG-3' (BamH1). The BamH1-digested fragment was ligated into pMal-cRI. The integrity of subcloning was verified by complementation assay as described above. The point mutation (S378A) was made by recombination PCR (33). 

A GmSerat2;1 cDNA containing a full-length open reading frame was cloned by RT-PCR using total RNA prepared from soybean culture cells and gene-specific primers. PCR products were cloned into either pdGN (34) for expression of carboxyl-terminal GFP fusion proteins or pdYN (J.-Y. Lee, unpublished data) for expression of C-terminal YFP fusion proteins under the control of the Cauliflower Mosaic Virus 35S promoter. For construction of pMal-cRI/GmSerat2;1, PCR was performed with primers YooP1 (5'-CGGAATTCATGAATGTTCTGGCTCTAGGGCG-3') and YooP2 (5'-GGATCCCTATATAACATAATCAGACCATG-3') as template. 

**Expression and Purification of Fusion Proteins** – Protocols for expression of recombinant proteins were described previously (35). For purification of MBP fusion proteins, bacterial cells were resuspended in extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 10 mM EDTA, 20 µg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 10 µg/ml leupeptin) and lysed by sonication. After clarification by centrifugation at 10,000 g for 20 min, the supernatant was passed through a 0.45 µm filter and loaded onto a column of amylose-agarose (New England BioLabs, Ipswich, MA) equilibrated with a binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl). After extensive washing with binding buffer, proteins were eluted with 10 mM maltose in binding buffer. Purified proteins were stored in 50% glycerol at -80 °C. The activity of MBP-GmSerat2;1 was stable at room temperature for several days, and small losses of activity (10-20%) were observed after several cycles of freezing and thawing. Inclusion of DTT and glycerol in the purification buffers did not affect yield, but appeared to help preserve activity during freezing and thawing. Expression and purification of His6-CDPK was described previously (31).

**Protein Kinase Assay** – CDPK activity assays were performed as described previously (31). **Phosphorylation of Serine Acetyltransferase** – Phosphorylation of SAT was performed on ice in 50 mM HEPES, pH 7.2, 10 mM MgCl₂, 1 mM EGTA, 1.2 mM CaCl₂ (omitted for calcium-free
reactions), 1 mM DTT, 0.05 mg/ml BSA, 1 mM \[^{32}P\]-ATP (~500 cpm/pmol) and 4 ng of His6-CDPK for every 1 \(\mu\)g of MBP fusion protein. The reactions were initiated by addition of ATP, and stopped by the addition of 4 volumes of stop buffer (50 mM HEPEs, pH 7.2, 10 mM EDTA, 5 mM EGTA). Aliquots of reactions were resolved on SDS-PAGE, and dried gels were exposed to X-ray film. To determine phosphorylation stoichiometry (moles of \(^{32}\)P incorporated per mole protein) protein bands were excised from gels and radioactivity was measured by liquid scintillation counting. To test the effect of phosphorylation on SAT activity, phosphorylation reactions were carried out as described above except without radioisotope, and aliquots were used to measure SAT activity.

**Activity Assays of Serine Acetyltransferase** – The radiometric assay was carried out in a 100 \(\mu\)l mixture that contained 50 mM Tris-HCl, pH 7.5, 1 mM acetyl-CoA, and 1 mM \(^3\)H-L-serine (1 \(\mu\)Ci), with or without 1 mM L-cysteine, and with indicated amounts of SAT, either untreated or phosphorylated by CDPK. The reaction was initiated by the addition of \(^3\)H-L-serine, incubated for various times at 30 °C, stopped by addition of 1/5 volume of concentrated ammonium hydroxide, and incubated an additional 60 min to ensure complete conversion of OAS to \(N\)-acetylserine (NAS) (36). An aliquot of the reaction mixture was passed over 0.5 ml of AG 50W-X8 resin (BioRad Laboratories, Hercules, CA) to resolve NAS from serine (37). NAS was eluted with 3 ml of water, and aliquots were analyzed by liquid scintillation counting. A blank reaction containing all components except acetyl-CoA was run in parallel and subtracted as a background reading. The identities of the reaction products, the completeness of the conversion of OAS to NAS, and the efficiency of NAS elution were examined by thin layer chromatography (TLC), ninhydrin staining, and autoradiography, using standard compounds as controls. TLC was performed on Cellulose 300, 100 micron TLC plates (Selecto Scientific, Inc., Suwanee, GA) and developed with butanol:water:acetic acid, 65:25:15 by volume.

Activities of full-length GmSerat2;1 constructs were determined by a spectrophotometric assay based on the absorbance of acetyl-CoA at 232 nM (\(\varepsilon = 4500\)). Reactions were carried out at room temperature (23 °C) in a volume of 1 mL containing 100 mM sodium phosphate buffer, pH 7.0, 0.1 mM acetyl CoA, 5 mM L-serine, various concentrations of L-cysteine, and 1 \(\mu\)g of SAT. Absorbance readings were collected every 3 sec for a period of 1 min during which time the reaction rate was linear.

Specific activities determined by the two assays differed (Tables 1 and 2), but the effects of mutations, phosphorylation, and cysteine on activity were consistent, regardless of the assay used.

**Antibodies** - SAT general antibody (gAb) was made with MBP-GmSerat2;1\(\Delta N\) as antigen by Cocalico Biologicals, Reamstown, PA. Antibodies precipitated from 10 ml antiserum by 50% saturated ammonium sulfate were dialyzed against TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl), and then loaded onto an affinity column of GmSerat2;1\(\Delta N\)-Sepharose. The resin was washed with 10 column volumes of TBS, and antibodies were eluted with 1 M Acetic Acid, 0.5 M NaCl, pH 2.0. Fractions with highest absorption readings at 280 nm were pooled, buffer-exchanged to TBS, concentrated, and lyophilized.

GmSerat2;1 C-terminal antibodies (cAb) were made by Cocalico Biologicals, with 20-mer peptides CPPNPIKLDKMP\(SFTMDHTS\) and CPPNPIKLDKMP\([pS]\)FTMDHTS as antigens. Peptides were synthesized by the Interdisciplinary Center for Biotechnology Research at the University of Florida, and included N-terminal cysteine residues used for coupling of the peptide to SulfoLink gel (Pierce Biotechnology, Rockford, IL), spacer proline residues, and the C-terminal phosphorylation site of GmSerat2;1 (residues 368-385). A 50% ammonium sulfate fraction of the antiserum dialyzed against antibody-binding buffer (10 mM Tris-HCl, pH 8.0, 1 mM NaF) and loaded onto dephospho-peptide column, and then flow-through fractions were loaded onto phospho-peptide column (P-column). The P-column was washed first with 10 column volumes of antibody binding buffer (10 mM Tris-HCl, pH 8.0, 0.5 M NaCl). Antibodies were eluted from P-column with 100 mM glycine, pH 2.0, buffer-exchanged to TBS, and concentrated. Unexpectedly, the purified antibodies recognized both dephospho- and phospho-MBP-GmSerat2;1\(\Delta N\) equally well.
Polyclonal antibodies that specifically recognized phosphorylated GmSerat2;1 (pAb), were made by Sigma-Genosys, The Woodlands, TX, using a 13 mer peptide, CPLDKMP[pS]FTMDH (residues 372-383 of GmSerat2;1 plus N-terminal CP). Antibodies were affinity purified on a column of immobilized phosphopeptide, however they also recognized dephosphoGmSerat2;1. To obtain the phospho-specific antibody (pAb) the latter antibodies were further purified by the same protocol used for purification of gAb, except that phospho-and dephospho-13 mer peptides were used for affinity purification. Aliquots of antibodies were stored at –20 °C.

Protein Extraction and Immunoprecipitation - Harvested soybean cells were homogenized in ice-cold extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 250 mM Sucrose, 2 mM DTT, 2 mM EDTA, 2 mM EGTA, 1 mM NaF, 1 mM Na2MoO4, 1 mM Na2VO3, 1 mM Microcystin LR, 1 mM PMSF, and complete protease inhibitor cocktail (Roche Diagnostics Corporation, Indianapolis, IN). Cell debris was pelleted by centrifugation for 60 min at 40,000 g, and proteins were precipitated from the supernatant by ammonium sulfate at 20 to 50% saturation. Protein pellets were resuspended in desalting buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM DTT, 1 mM NaF, 1 mM Na2MoO4, 1 mM Na2VO3). The solution was clarified by centrifugation and passed over a desalting column equilibrated with desalting buffer. The desalted fraction was supplemented with 1x complete protease inhibitor cocktail (Roche) and 1% NP-40 and pre-cleared of nonspecific proteins by incubating with Protein-G Sepharose (Sigma) for 2 h at 4 °C. Immunoprecipitation was carried out with 5 µg of antibodies for 2 h at 4 °C, with the same amount of total protein for each experiment, as determined according to Bradford using bovine serum albumin as standard. Protein-G Sepharose was added to bring down immunocomplexes. Protein G pellets were washed four times with desalting buffer, once with 50 mM Tris-HCl, pH 7.5, and then boiled for 5 min in 2x Laemmli SDS sample buffer. Proteins were resolved either in conventional 10% Laemmli SDS-PAGE gels using Tris-glycine buffer or in precast 10% Bis-Tris NuPAGE gels (Invitrogen, Carlsbad, CA) using MOPS running buffer.

Immunoaffinity Purification of GmSerat2;1 from Cell Extracts - A 20-50% ammonium sulfate fraction of cell extract was dissolved in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM DTT, 1 mM NaF, 1 mM Na2MoO4, and 1 mM Na2VO3, passed through a desalting column to exchange the buffer for Ag/Ab binding buffer (Pierce), and then loaded onto gAb-Sepharose. After the column was washed with 15 bed volumes of binding buffer, bound proteins were eluted using gentle Ag/Ab elution buffer (Pierce). Fractions with highest absorption readings at 280 nm were pooled, dialyzed against 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM DTT, and stored in aliquots at –20 °C.

Immunoblotting Analysis - Proteins blotted onto nitrocellulose membrane were detected by incubation with the indicated primary antibody and γ-chain-specific goat-anti-rabbit IgG conjugated to horseradish peroxidase (Sigma) as secondary antibody. Signals were visualized with Western SuperSignal Femto substrate (Pierce) and exposure to Biomax ML film (Kodak, Rochester, NY). For additional analysis of the same blot, antibodies were stripped off the membrane with Western Blot Restoring Buffer (Pierce) and probed with a different primary antibody. Signals on films were scanned, digitized, and, where indicated, quantified using NIH Image (http://rsb.info.nih.gov/nih-image). Calibration was done using known concentrations of His6-CDPKα. For quantification of phosphorylation and total protein of GmSerat2;1, signals were normalized to those measured for untreated cells, and presented as relative units.

Protein Phosphatase Assay - Resuspended 20-50% ammonium sulfate fractions from cell extracts were desalted on a gel filtration column equilibrated in phosphatase assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM DTT, EDTA-free complete protease inhibitor (Roche)). For protein phosphatase 1 (PP1) the assay buffer was supplemented with 200 µM Mn2+, and the PP1-specific inhibitor PPI-2 was added for some reactions. For alkaline phosphatase (AP), 1 mM Mg2+ and 0.1 mM Zn2+ were added to the reaction. Reactions proceeded for 10 min at 30 °C, and were stopped by the addition of Microcystin LR and EDTA to final concentrations of 1 µM and 1 mM, respectively. PP1, PP2A and PPI-2 were from
CalBiochem and AP from Sigma-Aldrich, St. Louis, MO. 

Subcellular localization - Constructs encoding GmSerat2;1 fused to the N-terminus of either GFP or YFP were introduced into Nicotiana benthamiana leaves or dark-germinated soybean radicles by microprojectile bombardment with a BioRad PDS-1000. A Zeiss LSM 510 NLO Multiphoton confocal microscope was used to collect images 12-24 h post-bombardment.

RESULTS

Identification of GmSerat2;1 by interaction cloning.

To identify potential substrates and/or interacting proteins of soybean CDPKs, we adapted a well-established interaction cloning protocol (29), which was instrumental in identification of proteins that interact with protein kinases (30,38). Screening of 150,000 pfu for binding to three 32P-labeled soybean CDPKs resulted in identification of eleven proteins that interacted with CDPK. To test whether these proteins were potential substrates for CDPK, phagemids were induced to express encoded proteins in E. coli, and bacterial extracts were subjected to phosphorylation assays. Interacting protein 3 (IP3) was not detectable by staining with Coomassie blue, but it was strongly labeled by 32P in phosphorylation assays (Supplementary Fig. S1) and it was chosen for further analysis.

A database search revealed that IP3 encoded a protein homologous to serine acetyltransferase. The full-length cDNA sequence (Fig. 1) was cloned using IP3 sequence and an EST clone (BM523465), which overlaps with the 5'-end of IP3. There is an in-frame stop codon (TAA) upstream of the putative start codon. The protein predicted from the full-length cDNA has a molecular mass of 42.5 kD. In sequence similarity analysis the deduced protein sequence clustered with Group 2 SATs (8), which includes AtSerat2;1, AtSerat2;2, and tobacco SATs 1 and 4 (Supplementary Fig. 2). The gene was named GmSerat2;1 in accordance with the nomenclature proposed by Kawashima et al. (8).

Five additional EST clones in the GenBank database, AI437954 and BM522920 from a root library, BI974912 from drought-stressed leaf tissue, BI424359 from cotyledons of 18-day-old seedlings, and BM893201 from cotyledons of etiolated seedlings, match exactly with regions of GmSerat2;1 cDNA sequence. These ESTs from various tissues suggest expression of GmSerat2;1 throughout the plant.

GmSerat2;1 is localized in the cytoplasm and with plastids.

Serine acetyltransferases are found in the cytoplasm, plastids, and mitochondria (8). To determine the subcellular localization of GmSerat2;1, full-length enzyme tagged C-terminally with either green fluorescent protein (FL-GFP) or yellow fluorescent protein (FL-YFP) was transiently expressed in leaf and root cells, respectively. GFP and YFP alone were found in the cytoplasm and nucleus, but not plastids (Fig. 2A and E). FL-GFP was found in the cytoplasm and organelles (Fig 2B). In high-resolution optical section images, these organelles were identified as chloroplasts (Fig. 2C and D), which showed superimposed signals from chlorophyll (red autofluorescence) and FL-GFP (green). Where the GFP and chlorophyll signals overlap, the color shifted to yellow. The magnified image of the chloroplast in Fig. 2D revealed a halo of green fluorescence surrounding a core of red fluorescence. This implies that GmSerat2;1 is located on the surface or in the intermembrane space of chloroplasts. In bean radicle cells, FL-YFP was found in the cytosol and plastids (Fig 2F).

GmSerat2.1 is phosphorylated on Serine 378 by CDPK.

Preliminary experiments indicated that GmSerat2;1 might be a potential substrate for CDPK (Supplementary Fig. S1 and data not shown). Sequence analysis of GmSerat2;1 suggested a potential phosphorylation site (Ser378) in the carboxyl terminus of GmSerat2;1 that matched the motif B-X-X-S/T (where B is a basic residue lysine or arginine, X is any residue, and S/T is serine or threonine) that is phosphorylated by CDPKs (39). This site was of particular interest, since bacterial SATs and plant SATs contain a regulatory site in their carboxyl termini (40-42). As shown in Supplementary Fig.
S3A, this motif is also present in SATs from several plant species, including chickpea, tobacco, potato, tomato, lettuce, and sunflower. Alignment of full-length protein sequences of SATs (Supplementary Fig. S2), showed that the two tobacco SATs having the predicted phosphorylation site clustered with GmSerat2;1 in Group 2. The Arabidopsis SATs in Group 2, AtSerat2;1 and AtSerat2;2 (Supplementary Fig. 3B) had some conserved elements of the phosphorylation motif with either Cys or Gly at the position of Ser378. None of the SATs in Groups 1 or 3 had any similarity to the phosphorylation motif.

To test whether the carboxyl-terminal site was the location of phosphorylation, GmSerat2;1 N, (encoded by clone IP3, and hereafter called ΔN) and full length GmSerat2;1 (hereafter called FL) were expressed in bacteria as fusion proteins with maltose-binding protein (MBP) at their amino termini and purified. No difference in the abilities of CDPKs α, β, and γ to phosphorylate a given protein, either ΔN or FL, was observed (data not shown). A phosphorylation stoichiometry of up to 0.95 mol phosphate per mol of ΔN was observed, and this agreed with the prediction of a single CDPK phosphorylation site in this protein (data not shown). To test whether Ser-378 was the phosphorylation site for CDPK, a mutant in which serine at this position was changed to alanine (GmSerat2;1ΔN/S378A, hereafter called ΔN/S378A) was also expressed and purified. During a 40-minute incubation with His6-CDPKα and [γ-32P]ATP, ΔN was phosphorylated to a stoichiometry of 0.7 mol P/mol protein, while negligible phosphorylation was detected for ΔN/S378A (Fig. 3A). No phosphorylation was detected for MBP alone (data not shown). To ask if additional CDPK phosphorylation sites were present in full length GmSerat2;1, equal amounts of ΔN, FL, and two phosphorylation site mutants, FL/S378A and FL/S378D (full-length GmSerat2;1 in which Ser378 was replaced by Asp), were phosphorylated by CDPKγ (Fig. 3B). In comparison to FL (lane 2) phosphorylation of the two phosphorylation site mutants was negligible (lanes 3 and 4), showing that Ser-378 is the only CDPK phosphorylation site in these proteins. FL was not phosphorylated as well as ΔN (lanes 2 and 5). Similar results were obtained when these proteins were phosphorylated by CDPKα (data not shown).

Phosphorylation of Serine 378 relieves feedback inhibition by cysteine.

Like bacterial and certain plant SATs (3,43), FL and ΔN were inhibited by cysteine (Fig. 3C and D). Both enzymes were inhibited with an IC₅₀ of 40 µM, showing that the N-terminal 49 residues do not contribute to cysteine inhibition. Since certain residues within the carboxyl termini of cysteine-sensitive SATs are required for the inhibitory effect of cysteine (40-42), we predicted that phosphorylation of Ser378 would block inhibition by cysteine.

To examine whether phosphorylation would alter the catalytic or regulatory properties of the enzyme, FL and ΔN were incubated with CDPKγ in kinase assay buffer for 30 min, then assayed for SAT activity in the presence and absence of cysteine. Incubation with CDPK increased the IC₅₀ for cysteine of FL and ΔN to 70 µM (Fig. 3D) and >150 µM (Fig. 3C), respectively. The relative effect of phosphorylation on the cysteine sensitivities of FL and ΔN enzymes correlated to their relative levels of phosphorylation (Fig. 3B).

Mutation of Ser378 to either Ala or Asp did not significantly affect specific activity in the absence of cysteine (Table 1 and Fig. 4), showing that these mutations did not affect the ability of the enzyme to catalyze the formation of OAS. However, these mutations had dramatically different effects on the sensitivity to inhibition by cysteine. Mutation of Ser 378 to Ala lowered the IC₅₀ for cysteine inhibition to 18 µM, whereas mutation to Asp rendered the enzyme insensitive to cysteine inhibition (Fig. 3E and F). Replacement of a phosphorylatable serine residue with aspartate introduces a negative charge that can mimic the effect of phosphorylation, and these data show that the S378D mutant mimics fully phosphorylated GmSerat2;1. Incubation of these mutants in the phosphorylation reaction, which results in negligible phosphorylation of either protein (Fig. 3A), had no effect on the IC₅₀ for Cys inhibition for either mutant (Fig. 3E and F). These data further support the conclusion that phosphorylation of Ser378 by CDPK blocks feedback inhibition by cysteine.
To investigate the relationship of phosphorylation stoichiometry to the degree of cysteine inhibition, ΔN and ΔN/S378A were incubated with His6-CDPKα in parallel reactions with either unlabeled ATP or [γ-32P]ATP. Samples were taken at several time points from respective reactions to determine both enzyme activity in the absence or presence of cysteine and the phosphorylation stoichiometry. As shown in Fig. 4, in the absence of cysteine, similar activity was detected for both ΔN and ΔN/S378A at each time point, and this activity was comparable to that detected in the control reactions (-) in which enzymes were not incubated with His6-CDPKα. These results show that phosphorylation itself did not significantly change the catalytic activity of the enzyme. However, in the presence of cysteine, ΔN incubated with CDPKα had greater activity than the control, and the activity increased with longer incubation time. In contrast, similar enzyme activities were obtained for ΔN/S378A reactions irrespective of the length of the incubation time. A plot of the activity of ΔN versus phosphorylation stoichiometry (Fig. 4C) shows a direct correlation between the relative SAT activity in the presence of cysteine and the enzyme’s phosphorylation state, demonstrating a direct relationship between phosphorylation of GmSerat2;1 by CDPK and insensitivity to feed back inhibition.

GmSerat2;1 is phosphorylated by an endogenous Ca2+-stimulated protein kinase.

To test whether GmSerat2;1 can be phosphorylated by endogenous kinases, a phosphorylation assay was performed with a soybean cell extract as the source of kinase activity. As shown in Supplementary Fig. S4A, phosphorylation of ΔN by endogenous kinases was greatly enhanced in Ca2+-containing buffer, while no Ca2+-stimulated increase in phosphorylation of ΔN/S378A was observed. The very weak phosphorylation of ΔN/S378A and of ΔN in the Ca2+-free buffer suggests Ca2+-independent phosphorylation of GmSerat2;1 at a site other than Ser-378. The ability of endogenous protein kinases to phosphorylate ΔN but not ΔN/S378A in a Ca2+-dependent manner supports the hypothesis that GmSerat2;1 is phosphorylated on Ser-378 by CDPK in vivo.

To study the phosphorylation of GmSerat2;1 in vivo, antibodies that specifically recognize the phosphorylated enzyme or that recognize both phospho- and dephospho-GmSerat2;1 were made. General antibody (gAb), C-terminal antibody (cAb), and phospho-specific antibody (pAb) were produced using GmSerat1;2-ΔN, C-terminal peptide, and phosphorylated C-terminal-peptide, respectively. Western analysis showed gAb recognized both dephosphorylated and phosphorylated ΔN (Fig. 5A), while phospho-specific antibody (pAb) recognized only ΔN phosphorylated by CDPK in vitro (Fig. 5B). Pre-absorption of pAb with the 13 mer phosphopeptide corresponding to the C-terminus of GmSerat2;1, but not with the 13 mer dephosphopeptide, completely blocked the recognition of phosphorylated ΔN by pAb (Fig. 5C), indicating pAb is specific for the phosphorylation site containing phosphoSer378.

Western blot analysis with these antibodies was performed to test whether GmSerat2;1 is phosphorylated in vivo (Fig. 5D). Due to the low abundance of GmSerat2;1 in vivo (data not shown), it was necessary to enrich the enzyme by immunoaffinity chromatography with Sepharose-conjugated gAb prior to analysis. All three GmSerat2;1 antibodies, gAb, cAb, and pAb, recognized a protein of 42 kD, the size predicted for endogenous GmSerat2;1. Preincubation of pAb with the 13-mer phosphopeptide of GmSerat2;1 (pAb/P), but not with the 13 mer dephosphopeptide (pAb/D), abolished the recognition of GmSerat2;1 by pAb, indicating that it is phosphorylated in vivo at the predicted phosphorylation site, Ser-378. Besides the 42 kD GmSerat2;1 band, gAb recognized an additional 29 kD protein. This protein may be the soybean SAT isoform reported by Chronis & Krishnan (2004).

PP1 dephosphorylates GmSerat2;1.

To gain insight into which phosphatase is responsible for the dephosphorylation of GmSerat2;1, cell extracts were treated with three different protein phosphatases in vitro. Immunoprecipitation (IP) was carried out with gAb, and then IP pellets were analyzed by western
blot with pAb as well as gAb. As seen in Supplementary Fig. S4C, PP1, but not PP2A or alkaline phosphatase, dephosphorylated GmSerat2;1. PP1 was unable to dephosphorylate GmSerat2;1 when PPI-2, a specific peptide inhibitor to PP1, was included in the reaction, indicating phosphatase activity of PP1 was specific to dephosphorylation of GmSerat2;1.

**Hydrogen peroxide induces the synthesis and phosphorylation of GmSerat2;1 in vivo.**

Exogenous application of H$_2$O$_2$ is known to trigger a rise in [Ca$^{2+}$]$_{cyt}$ (21,24), and thus potentially activates CDPKs (35). To ask whether changes in the abundance and phosphorylation state of GmSerat2;1 occur in response to oxidative stress in vivo, we used antibodies to analyze levels of phosphorylated and total GmSerat2;1 following exposure of soybean cells to exogenous H$_2$O$_2$. As seen in Fig. 6, the levels of both total and phosphorylated GmSerat2;1 protein increased following H$_2$O$_2$ treatment. After 60 min of H$_2$O$_2$ treatment, the level of total GmSerat2;1 was ~3-fold higher than that in control cells, and it remained elevated for at least 4 h after oxidant challenge. Analysis of the ratio of phosphorylated to total GmSerat2;1 protein increased between 5 and 30 min the fraction of phosphorylated protein increased relative to the total. On average, a 2- to 5-fold increase in the ratio was evident as soon as 5 min following H$_2$O$_2$ treatment. After 30 min the fraction of phosphorylated enzyme declined as the total protein level increased, but the fraction of phosphorylated protein present at 240 min post H$_2$O$_2$ treatment was nearly double that present at the beginning of the experiment.

**Agents that perturb Ca$^2+$ signaling block phosphorylation of GmSerat2;1 in vivo.**

To investigate whether calcium is involved in the phosphorylation of GmSerat2;1 in vivo following oxidative stress, three different classes of inhibitors that perturb Ca$^{2+}$ signaling were analyzed in combination with 60 min H$_2$O$_2$ treatment (Fig. 7A). A 1 h incubation with 2 mM EGTA, which chelates extracellular Ca$^{2+}$, greatly reduced GmSerat2;1 phosphorylation, but had little effect on the total protein level (Fig. 7A), suggesting extracellular Ca$^{2+}$ is important for maintaining the basal level of GmSerat2;1 phosphorylation under normal growth conditions. The ability of H$_2$O$_2$ to induce GmSerat2;1 phosphorylation in EGTA-pretreated cells to the level equivalent to that in control cells suggests intracellular Ca$^{2+}$ may be more important than extracellular Ca$^{2+}$ for the induction. Preincubation of cells with A9C or NA, which indirectly inhibit intracellularly derived Ca$^{2+}$ in plant cells (44), interfered with both protein synthesis and phosphorylation induced by H$_2$O$_2$. Similar results were observed with a 5-min H$_2$O$_2$ treatment (Supplementary Fig. S5).

The accumulation of GmSerat2;1 in response to hydrogen peroxide treatment results from increased protein synthesis.

To assess whether the increased GmSerat2;1 protein level, observed 1 h after H$_2$O$_2$ treatment, was due to an increase in protein synthesis or a decrease in protein degradation, we treated cells with cycloheximide (CHX), a protein synthesis inhibitor, prior to H$_2$O$_2$ treatment. Fig. 7C shows that pretreatment of cells with CHX blocked the H$_2$O$_2$-induced increase in levels of total GmSerat2;1 protein (compare the total protein levels in the two 60-min samples), indicating that exposure to H$_2$O$_2$ induced synthesis of GmSerat2;1 rather than suppressing its degradation in soybean cells.

**Phosphorylation does not affect the stability of GmSerat2;1.**

A change in the phosphorylation state of a protein can lead to its proteosome-mediated protein destruction (45,46). To ask if inhibition of phosphorylation would increase GmSerat2;1 stability, and to also ask whether phosphorylation events are involved in the signaling pathway leading to H$_2$O$_2$-induced GmSerat2;1 synthesis, cells were treated with staurosporine (ST), a general protein kinase inhibitor, prior to oxidant challenge and levels of phosphorylated and total GmSerat2;1 were determined. As seen in Fig. 7D, while ST pretreatment almost completely abolished phosphorylation, it had little effect on the increased accumulation of total GmSerat2;1 protein, which was equivalent to that in cells without ST pretreatment. This observation
suggests that phosphorylation events are not required for H$_2$O$_2$-induced GmSerat2;1 synthesis. Consistent with the idea that phosphorylation of GmSerat2;1 is not associated with its degradation, levels of both relative phosphorylation and total protein increased in cells following H$_2$O$_2$ treatment (Fig. 6). Most importantly, in experiments in which protein synthesis was inhibited by cycloheximide, no decrease in the level of GmSerat2;1 was observed upon H$_2$O$_2$-induced increase in phosphorylation (compare lanes 4 to 6 in Fig. 7C).

**DISCUSSION**

In a screen for proteins that interact with CDPKs, we identified the serine acetyltransferase GmSerat2;1. We demonstrated that GmSerat2;1 is a substrate for CDPK in vitro (Fig. 3-4), and that it is phosphorylated in vivo in response to oxidative stress (Fig. 6). While phosphorylation has no direct effect on the activity of the enzyme, it blocks inhibition of activity by cysteine (Fig. 3-4), the end product of the biosynthetic pathway in which serine acetyltransferase participates. Our observations that protein kinase activity present in soybean cell extracts phosphorylates GmSerat2;1 (Supplementary Fig. S4), and that the phosphorylation is greatly stimulated by Ca$^{2+}$ (Fig. 7) are consistent with the idea that GmSerat2;1 activity is regulated by CDPK in vivo.

GFP-tagged GmSerat2;1 localized in the cytoplasm and with plastids (Fig. 2). This result must be interpreted with caution, because of possible mislocalization due to overexpression of the GFP-tagged enzyme under the control of the 35S promoter. However, the result is consistent with the dual localization Arabidopsis SAT-p (AtSerat2;1) (9). This SAT was localized with plastids in 4-week-old leaves, but found predominantly in the cytoplasm in 6-week-old leaves. Localization of this SAT to plastids was questioned by Droux (2), since in vitro-translated protein (then called SAT5) was not imported into chloroplasts (47). Localization of the enzyme to the outside of the chloroplasts could explain both sets of observations. Interestingly, SAT-p is the only one of the five Arabidopsis SATs that is associated with plastids (8,9). CDPKs are found in the cytoplasm, plasma membrane, endoplasmic reticulum, and peroxisomes (48). Cytosolic CDPKs would have access to SAT located in the cytoplasm or on the surface of plastids.

The IC$_{50}$ for cysteine inhibition of GmSerat2;1 is 40 µM, which places it in the class of SATs that have intermediate sensitivity to cysteine. Other SATs in this class are *Allium tuberosum* ASAT5 (IC$_{50}$ 49 µM) (49) and SAT1 (IC$_{50}$ 40 µM) and SAT7 (IC$_{50}$ 30 µM) from *Nicotiana tabacum* (3). SATs that are highly sensitive to cysteine are *Arabidopsis thaliana* AtSerat1;1 (IC$_{50}$ 1.8 µM) (9) and AtSerat3;2 (0.8 µM) (8), *Citrus vulgaris* SAT (2.9 µM) (50), *Allium cepa* SAT (3.1 µM) (51), *Spinacia oleracea* SAT (7.6 µM) (52), *Pisum sativum* SAT (12 µM) (2) and *Glycine max* SSAT1 (a Group 1 SAT; 4 µM) (53).

The cellular concentration of cysteine is thought to be 2-10 µM (54). The IC$_{50}$s of the intermediate sensitivity class of SATs (30-50 µM) are high in comparison to this value, and this difference casts some doubt on the importance of the intermediate sensitivity class in regulating cysteine in vivo; however, the variation in cysteine concentration in the cytosol, mitochondria, plastids and vacuole is not known, so the actual concentration of cysteine experienced by SATs in each compartment is not known. Experiments comparing the effect of tobacco SATs on the production of cysteine in bacteria suggest the potential of various sensitivity classes of SATs to limit cysteine synthesis in planta. When the endogenous SAT (CysE) of *E. coli* (IC$_{50}$ 1 µM) was replaced by tobacco SAT1 (IC$_{50}$ 40 µM) or SAT7 (IC$_{50}$ 30 µM) cysteine accumulation increased 3- to 4-fold, whereas replacement by SAT4, which is not inhibited by cysteine, supported a 50-fold increase in cysteine (55). These results suggest that intermediate sensitive forms of SAT support only slightly higher production of cysteine than do highly sensitive SATs, and that they limit cysteine synthesis relative to SATs that are not inhibited by cysteine. Our data show that phosphorylation of GmSerat1;2 converts it from an intermediately sensitive to an insensitive SAT, which would increase the accumulation of cysteine.

Analysis of plant SAT sequences available in electronic databases (Supplementary Fig. S3) shows that, in addition to soybean, species from several families of angiosperms and gymnosperms...
have SATs in which the C-terminal phosphorylation site is conserved. Interestingly, SATs from Arabidopsis and rice lack this site, thus phosphorylation of SAT is not universal. It has been noted that there is species variation in cysteine synthesis and sulfur metabolism (10), and now the regulation by phosphorylation of SATs from a subset of plants adds to the complexity. The observed variation shows the importance of studying the regulation of cysteine synthesis in plants on a species-to-species and organelle-to-organelle basis.

The phosphorylation site in GmSerat2;1 identified in this study, serine 378, is located close to the site found to be critical for feedback inhibition of SATs from watermelon (41,42) and Arabidopsis (56). Substitution of a single amino acid residue, either Gly 277 to Cys or Met 280 to Ile, in the C-terminal region of watermelon SAT causes a significant decrease in its sensitivity to cysteine inhibition (41,42). The comparable residues in GmSerat2;1 are Ser 378 and Met 381 (Supplementary Fig. S3B). The change of serine 378 to alanine or aspartic acid in GmSerat2;1 did not affect its catalytic activity, but mutation to aspartic acid mimicked phosphorylation and rendered the enzyme insensitive to feedback regulation by cysteine. In the crystal structure of cysteine-inhibited SAT from Haemophilus influenzae (43), residues of the C-terminus contact cysteine, which is bound at the active site. Introduction of negative charge into the C-terminus of SATs from plants and bacteria are important for not only feedback inhibition by cysteine, but also for the binding of SAT to OAS-TL (3,6,43,57,58). The observation that cysteine causes the complex between Cys-inhibited SAT and OAS-TL to dissociate (2), suggests a model in which the C-terminus can be involved in binding to either OAS-TL or to cysteine, but not both. The dual function of the SAT C-terminus is supported by crystal structures of bacterial SAT (43), described above, and OAS-TL. In the structure of E. coli OAS-TL crystallized with the C-terminal peptide from H. influenzae SAT, the SAT peptide is located in the active site of OAS-TL (58), and this interaction provides the basis for inhibition of OAS-TL in the cysteine synthase complex (2).

Addition of H₂O₂ to soybean cells stimulated phosphorylation and synthesis of GmSerat2;1 within 5 min of treatment (Fig. 6). The timing of this response is consistent with the observation that treatment of tobacco cells and Arabidopsis seedling with H₂O₂ resulted in a transient increase in cytoplasmic calcium within 1 min of treatment (21,24). Experiments with pharmacological effectors A9C and niflumic acid (Fig. 7) suggested that internally derived calcium was required for both rapidly induced GmSerat2;1 phosphorylation and increased protein synthesis in the late phase of the response to oxidant challenge. The sites of action of these agents in plant cells are not well defined, so these results should be viewed as tentative. Under normal culturing conditions, GmSerat2;1 exhibited a basal level of phosphorylation, which was greatly diminished by treatment with EGTA (Supplementary Fig. S5 and Fig. 7).

Based on data presented in this paper we propose a model for GmSerat2;1 regulation (Fig. 8). Signals such as abiotic or biotic stress increase intracellular free calcium (21,23-26,44,59,60), which in turn activates CDPK to phosphorylate and increases synthesis of GmSerat2;1. This two-fold response upon oxidant challenge provides a mechanism by which cells can respond quickly to H₂O₂ exposure through desensitized GmSerat2;1 to avoid the depletion of the cysteine pool, and then signal for more GmSerat2;1 protein to be synthesized to relieve a sustained stress condition. CDPK is able to couple Ca²⁺-signaling to the defense response by regulating a particular target.

The increase in the activity of GmSerat2;1 would support production of glutathione (20) or, in legumes homoglutathione. Environmental conditions such as oxidative stress and exposure to heavy metals can lead to the accumulation of (homo)glutathione and phytochelatins, which respectively scavenge excess reactive oxygen species and chelate metals that are toxic to the cells (61). Considerable increases in the pool size of glutathione have been reported in plants under adverse environmental stresses or challenge by pathogens (11). The increased biosynthesis of these cysteine-rich compounds puts an increased demand on cysteine synthesis, and the coordinated increases in glutathione level were observed when
cysteine contents were increased artificially by supplying sulfate (62), fumigation of H₂S (17,63), or by direct feeding of cysteine itself (64,65). These data suggest that cysteine availability plays an important role in determining glutathione content through kinetic limitation of the rate of its synthesis. The work reported here shows that in soybean cysteine synthesis in response to stress is regulated by synthesis and phosphorylation of GmSerat2;1.

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**FOOTNOTES**

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3The abbreviations used are: SAT, serine acetyltransferase; OAS, O-acetylserine, OAS-TL, O-acetylserine (thiol)lyase; IP3, clone encoding interacting protein 3; GmSerat2;1, Glycine max serine acetyltransferase 2;1; CDPK, calcium-dependent protein kinase; MBP, maltose-binding protein; PP1, protein phosphatase 1; GSH, glutathione; IP3, interacting protein 3; kD, kilodalton; EST, expressed sequence tag; GFP, green fluorescent protein; YFP, yellow fluorescent protein; FL, full-length Glycine max serine acetyltransferase 2;1; ΔN, amino-terminal deleted version of GmSerat2;1 encoded by IP3; gAb, general antibody; cAb, C-terminal antibody; pAb, phospho-specific antibody; IP, immunoprecipitation; RT-PCR, reverse transcriptase polymerase chain reaction; EGTA, ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid; A9C, anthracene-9-carboxylate; NA, niflumic acid; CHX, cycloheximide; ST, staurosporine; PCR, polymerase chain reaction; NAS, N-acetylserine; TLC, thin layer chromatography; NC, nitrocellulose

FIGURE LEGENDS

Fig. 1. Assembled nucleotide and deduced amino acid sequence of GmSerat2;1. An in-frame stop codon TAA at 5'-end untranslated region is underlined. The solid line with arrow designates the starting nucleotide of the original clone IP3, and the dashed line with arrow denotes the first amino acid of GmSerat2;1ΔN that was fused to MBP in the ΔN clone. The carboxyl-terminal CDPK phosphorylation site is boxed, with the serine at position 378 being the phosphorylated residue.

Fig. 2. Localization of GmSerat2;1 in the cytoplasm and plastids. Constructs encoding GmSerat2;1 fused to the N-terminus of either GFP or YFP were transiently expressed in the indicated tissues and visualized 12-24 h post-bombardment. (A – D) Nicotiana benthamiana leaves. (E & F) Bean radicles. (C) A higher resolution optical section of (B) with dual GFP (green) and chlorophyll (red) channels. (D) A magnified image of highlighted area in (C). Note that the autofluorescent chlorophyll on the top left of panels C and D are from a neighboring guard cell and that the orange colors of organelles resulted from the overlap of green and red fluorescence. Images are representative of > 30 cells expressing individual plasmids and three independent bombardment experiments of each construct. Bars, 10 μm.

Fig. 3. Phosphorylation and activity of GmSerat2;1 and mutant proteins. A. Either ΔN or ΔN/S378A was incubated on ice with His6-CDPKα plus [γ-32P]-ATP in Ca2+-containing assay buffer for the indicated times (minutes). Samples taken at the indicated time points were resolved on 10% SDS-PAGE (upper panel) and analyzed by autoradiography (lower panel). Bands corresponding to GmSerat2;1 proteins were cut from the gel and 32P incorporation was determined by scintillation counting, and phosphorylation stoichiometry (mole %) was calculated. Additional His6-CDPKα protein was added to the reactions 30 minutes following the initiation of the phosphorylation. B. One microgram of protein (Lane 1, MBP; 2, MBP-FL; 3, MBP-FL/S378A; 4, MBP-FL/S378D; 5, MBP-ΔN) was incubated for 30 min with 0.1 μg of CDPKγ in phosphorylation buffer with [γ-32P]ATP. Proteins were resolved on an SDS-PAGE gel (left panel), and the gel was autoradiographed (right panel). C-F. Effect of cysteine on the activity of
unphosphorylated and phosphorylated GmSerat2;1 and mutants. One microgram of the indicated enzymes was incubated for 30 min with or without 0.1 µg of CDPKγ in phosphorylation buffer with ATP. Following the phosphorylation reaction proteins were tested by the spectrophotometric assay for SAT activity in the presence of various concentrations of cysteine. Data are the averages and standard deviations for assays performed in triplicate. Dose-response curves for the effect of cysteine on the indicated enzymes incubated without and with CDPK.

**Fig. 4.** Phosphorylation desensitizes MBP-GmSerat2;1 ΔN (ΔN) from feedback inhibition by cysteine. Either MBP-ΔN (ΔN) (A) or MBP-ΔN/S378A (ΔN/S378A) (B) was pre-incubated on ice with His6-CDPKα and ATP in Ca2+-containing buffer for indicated period of time (-, no pre-incubation; 0, pre-incubation in Ca2+-free buffer), and then samples were analyzed for phosphorylation stoichiometry as well as catalytic activity in the absence or presence of 1 mM L-cysteine. SAT activity was measured in the radiometric assay. Error bars represent standard errors of duplicate reactions. The maximal activity (100%) was 73.7 µmol NAS / mg protein / min, measured in the absence of cysteine. C. Correlation between activity and phosphorylation state. The activities of ΔN pre-phosphorylated to various degrees were determined in the presence of cysteine and plotted as a function of the phosphorylation stoichiometry of the enzyme. An enzyme activity of 100% is equivalent to the activity of ΔN assayed in the absence of cysteine. The line drawn on the graph was determined by linear regression.

**Fig. 5.** GmSerat2;1 is phosphorylated in vivo. A-C. Characterization of GmSerat2;1 antibodies. MBP-GmSerat2;1/ΔN fusion proteins, either unphosphorylated (ΔN) or phosphorylated by CDPKα in vitro (ΔNp), were analyzed by western blot with general antibodies gAb1 (A), untreated phosphorylation state-specific antibodies pAb (B), or pAb pretreated with either GmSerat2;1 phospho-peptide or GmSerat2;1 dephospho-peptide (C). D. Proteins purified from soybean cell extract using an affinity column conjugated with gAb1 (see Materials and Methods) were analyzed by western blot with different antibodies: C, control with secondary antibody alone; gAb, general GmSerat2;1 antibody; cAb, C-terminal GmSerat2;1 antibody; pAb -, untreated phospho-specific antibody; pAb P; phospho-specific antibody pretreated with GmSerat2;1 phospho-peptide CPLDKMP[pS]FTMDH; pAb D; phospho-specific antibody pretreated with GmSerat2;1 dephospho-peptide CPLDKMPSFTMDH. The asterisk designates a nonspecific protein that is immunostained by the secondary antibody.

**Fig. 6.** Time course of GmSerat2;1 in response to H2O2 treatment. A. Cultured soybean cells were collected at indicated time following treatment with 0.5 mM H2O2. gAb immunoprecipitates were analyzed by western blot with pAb and gAb to determine the levels of phosphorylated (phospho) and total (total) GmSerat2;1, respectively. These results are representatives of two experiments. B. Quantitative analysis of data shown in A. The plot shows the total protein level relative to that of untreated cells (circles), and ratio of phosphorylated to total GmSerat2;1 protein normalized to the ratio for untreated cells (squares).

**Fig. 7.** Effect of inhibitors of protein synthesis and phosphorylation and Ca2+ antagonists on H2O2-induced phosphorylation of GmSerat2;1. A. External Ca2+ is not required for GmSerat2;1 phosphorylation or synthesis. Soybean cells were treated with 2 mM EGTA for 1 h prior to a two-hour treatment with 0.5 mM H2O2. B. Internal Ca2+ is required for H2O2-induced GmSerat2;1 synthesis. Soybean cells were pretreated for 15 minutes with internal Ca2+ inhibitors, 200 µM A9C or 50 µM NA, or DMSO (solvent), prior to a one-hour treatment with 0.5 mM H2O2. C. Soybean cells were treated with 30 mM cycloheximide (CHX) for 1 h prior to the application of 0.5 mM H2O2. Cells were harvested after an additional 30 or 60 minutes. D. Decoupling of H2O2-induced synthesis of GmSerat2;1 from its phosphorylation. Soybean cells were pretreated with 2 µM staurosporine (ST, a general protein kinase inhibitor) or DMSO (solvent of ST) for 15 minutes prior to a one-hour treatment with 0.5 mM H2O2. In
all experiments proteins were immunoprecipitated with gAb and analyzed by western blot with pAb and gAb to determine the levels of phosphorylated (phospho) and total (total) proteins, respectively.

**Fig. 8.** A proposed working model. GmSerat2;1 protein exists in two states; a dephosphorylated state, which is sensitive to the feedback inhibition by cysteine, and an active phosphorylated state. Oxidative stress induces a rapid phosphorylation of GmSerat2;1 to prevent the potential depletion of cysteine pool, and an increase in total protein to tolerate the sustained stress through the potential increase of GSH. Externally derived Ca^{2+} contributes to retaining GmSerat2;1 at a basal level of phosphorylation, while Ca^{2+} released from internal storage sites such as ER, vacuoles, mitochondria and chloroplasts are required for the activation of CDPK and other Ca^{2+}-modulated proteins such as CaMs, which may regulate the transcription or translation of GmSerat2;1 in response to oxidative stress.
Table 1. SAT Activity Measured by the Spectrophotometric Assay

| Enzyme         | Activity<sup>a</sup> (µmol min<sup>-1</sup> mg<sup>-1</sup>) | Standard Deviation | N<sup>b</sup> |
|----------------|------------------------------------------------------------|--------------------|---------------|
| MBP-AN         | 14.2                                                       | 0.6                | 4             |
| MBP-FL         | 10.3                                                       | 2.3                | 5             |
| MBP-FL/S378A   | 9.8                                                        | 1.7                | 4             |
| MBP-FL/S378D   | 11.8                                                       | 3.0                | 5             |

<sup>a</sup>Activity was determined from the disappearance of acetyl Co-A measured by the change in absorbance at 232 nm and calculated using a molar extinction coefficient of 4500.

<sup>b</sup>Number of independent determinations of activity. At least two preparations of each enzyme were used.
Table 2. SAT Activity Measured by the Radiometric Assay

| Enzyme          | Treatment | Activity\(^a\) (µmol min\(^{-1}\) mg\(^{-1}\)) | Standard Deviation | N\(^b\) |
|-----------------|-----------|-----------------------------------------------|-------------------|--------|
| MBP-ΔN          | No Cys    | 74.18                                         | 0.56              | 3      |
| MBP-ΔN          | 1 mM Cys  | 12.18                                         | 0.98              | 3      |
| MBP-ΔN/S378A    | No Cys    | 73.64                                         | 0.80              | 3      |
| MBP-ΔN/S378A    | 1 mM Cys  | 16.45                                         | 1.35              | 3      |

\(^a\) Activity was determined by measuring the amount of [\(^3\)H]-N-acetylserine formed from [\(^3\)H]-serine.

\(^b\) Number of independent determinations of activity.
Fig 1, Liu et al., 2006
Figure 3, Liu et al., 2006

Graphs A and B illustrate the effect of cysteine concentration on the relative activity of various enzymes. Graph C shows the relative activity of ΔN/S378A and ΔN enzymes with and without CDPK. Graph D compares the activity of FL and FL/S378A enzymes under the same conditions. Graph E and F depict the activity of FL/S378A and FL/S378D enzymes, respectively.
Fig. 4, Liu et al., 2006

A. ΔN

SAT Activity (µM min⁻¹ mg⁻¹)

- Cys  + Cys

Pre-incubation with CDPKα (min)

0  10  30  40

B. ΔN/S378A

SAT Activity (µM min⁻¹ mg⁻¹)

- Cys  + Cys

Pre-incubation with CDPKα (min)

0  10  30  40

C. Relative Activity (%)

Phosphorylation Stoichiometry (%)

0  20  40  60  80
Fig. 5, Liu et al., 2006
Fig. 6, Liu et al., 2006

A

B
Fig 7, Liu et al., 2006

A

EGTA
H$_2$O$_2$
- - + + +

phospho

total

B

- - A9C NA DMSO Inhibitor
H$_2$O$_2$
- + + + +

phospho

total

C

- - - - 0 30 60 0 30 60

phospho

total

D

- - ST DMSO Inhibitor
H$_2$O$_2$ (60 min)
- + + + +

phospho

total
Calcium-regulated phosphorylation of soybean serine acetyltransferase in response to oxidative stress
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