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Subcellular Location of *O*-Acetylserine Sulfhydrylase Isoenzymes in Cell Cultures and Plant Tissues of *Datura innoxia* Mill.[1]

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***O*-Acetylserine sulfhydrylase (OASS; EC 4.2.99.8) catalyzes the formation of L-cysteine from *O*-acetylserine and inorganic sulfide. Three OASS isoenzymes that differ in molecular mass and subunit structure are present in shoot and root tissues and in cadmium-resistant and cadmium-susceptible cell cultures of *Datura innoxia* Mill. Different OASS forms predominate in leaves, roots, and suspension-cell cultures. To determine the subcellular location of the OASS isoenzymes, purified mitochondria, chloroplasts, and cytosolic fractions from protoplasts were obtained. The isoenzymes are compartmentalized in *D. innoxia* cells, with a different isoenzyme predominant in the chloroplast, cytosol, and mitochondria, suggesting that they serve different functions in the plant cell. The chloroplast form is most abundant in green leaves and leaf protoplasts. The cytosolic form is most abundant in roots and cell cultures. A mitochondrial form is abundant in cell cultures, but is a minor form in leaves or roots. Cadmium-tolerant cell cultures contain 1.8 times as much constitutive OASS activity as the wild-type cell line, and 2.9 times more than the cadmium-hypersensitive cell line. This may facilitate rapid production of glutathione and metal-binding phytochelatins when these cultures are exposed to cadmium.**

OASS (EC 4.2.99.8) produces L-Cys from sulfide and *O*-acetylserine, and thus is responsible for the final step in sulfur assimilation into organic molecules in plants. All plant metabolites that contain reduced sulfur are derived originally from the Cys produced by OASS. This enzyme is important in the regulation of sulfur uptake and in making available reduced sulfur (Cys) for various cellular functions.

Reduced sulfur is required for a number of functions critical to plant growth and development (Rennenberg, 1984; Rennenberg et al., 1990; Schmidt and Jäger, 1992). As Cys, it is required for the biosynthesis of several other primary metabolites such as Met. Reduced sulfur is also essential for the biosynthesis of coenzymes and as a component of plant membranes. Our research has focused on its role in the synthesis and metabolic activity of glutathione and its derivatives, compounds that protect plants against oxidative damage caused by a number of abiotic and biotic factors, including heavy-metal toxicity (Rauser, 1990; Jackson et al., 1992; Robinson et al., 1993).

Three isoenzymes of OASS from *Datura innoxia* Mill. cell-suspension cultures differing in size and subunit structure have been purified and characterized (Kuske et al., 1994). The existence of these three physically distinct forms suggests that the enzymes might be localized in different cellular compartments, and that they may provide Cys for different metabolic processes. To determine their subcellular location, we examined their presence and relative abundance in tissues, heterotrophic cell-suspension cultures, and isolated chloroplasts, mitochondria, and protoplasts from *D. innoxia* leaves and cell-suspension cultures.

MATERIALS AND METHODS

Plants and Cell-Suspension Cultures

Datura innoxia Mill. plants were grown from seed at 23 to 28°C and 14-h day length under a combination of lamps (Growlux and Growlux Wide Spectrum 75-W bulbs, Sylvania). $Cd300^R$, WDI, and $Cd300^S$ *D. innoxia* cell-suspension cultures were grown in darkness at 30 to 33°C in a modified B5 medium as described by Jackson et al. (1984). Suspension-culture cells for protein extraction were grown as 100-mL batch shake cultures in 500-mL DeLong flasks (Bellco Glass, Vineland, NJ). The cadmium-tolerant cells are able to grow continuously in medium containing 250 mM $CdCl_2$, whereas the sensitive cell lines die within 48 h following exposure to this concentration.

Soluble Protein Extracts from Plants and Cell-Suspension Cultures

Young, fully expanded leaves, young roots, or cells grown in suspension culture were used for protein isolation. Plant tissues were cut into 1-cm pieces and ground in liquid nitrogen to a fine powder. Cells from the suspension culture were collected by centrifugation at 1,000*g* for 1 min

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Abbreviations: $Cd300^R$, cadmium-tolerant cell culture; $Cd300^S$, cadmium-hypersensitive cell culture; OASS, *O*-acetylserine sulfhydrylase; WDI, wild-type cell line.

using a swinging-bucket centrifuge (International Equipment, Needham Heights, MA), washed with an equal volume of protein extraction buffer (30 mM Tris, pH 7.8, 10 mM 2-mercaptoethanol), and pelleted again by centrifugation. Pelleted, rinsed cells were quickly frozen in liquid nitrogen and ground to a fine powder. The powder from either plant tissues or cells was suspended in 50°C extraction buffer and cooled in an ice-water bath. The brei was centrifuged at 14,000*g* for 20 min and the supernatant was collected. This supernatant constituted the crude soluble protein extract.

D. innoxia cells from $Cd300^R$ exposed for 24 h to 250 or 0 μM $CdCl_2$, WDI, and $Cd300^S$ were harvested and the soluble proteins obtained as described above. The OASS forms were partially purified. Granular ammonium sulfate was slowly added to the supernatant with stirring to 20% saturation and the precipitate was collected by centrifugation at 21,000*g* for 20 min. The pellet was discarded and the supernatant was slowly brought to 80% ammonium sulfate saturation. The precipitated protein was pelleted by centrifugation at 21,000*g* for 20 min and gently suspended in 20 to 60 mL of a solution containing 60 mM Tris, pH 7.8, 10 mM 2-mercaptoethanol, and 10% glycerol. Ten- to 12-mL aliquots of the protein sample were applied to 100 × 2.5 cm columns (Sephacryl 200 HR, Pharmacia) equilibrated in the same buffer. OASS-active fractions eluted as a broad single peak following the void volume peak that contained most of the protein. Fractions with OASS activity were pooled and concentrated by filtration using a 30-kD exclusion filter (Amicon, Beverly, MA).

Chloroplast Isolation and Purification

Chloroplasts were purified from 150 g of *D. innoxia* leaf tissue using a method adapted from Bard et al. (1985). Leaves were homogenized in a blender (Waring) at 4°C in 800 mL of chloroplast extraction buffer (330 mM mannitol, 50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% defatted BSA, 10 mM 2-mercaptoethanol, and 1 mM $MgCl_2$), and the brei was filtered through two layers each of cheesecloth and Miracloth (Calbiochem). Chloroplasts were pelleted by centrifugation at 1400*g* for 10 min, suspended in 35 mL of chloroplast extraction buffer, and purified on field-formed gradients (Percoll, Pharmacia) (Schmitt et al., 1974). Gradient-purified, intact chloroplasts were washed three times in chloroplast-extraction buffer (minus the BSA), and the final pellet was suspended in 50 mM Tris, pH 8.0, 2 mM $MgCl_2$, and 1 mM DTT. Phase-contrast microscopy was used to ensure that the purified chloroplasts were intact.

Chloroplasts were lysed by three 10-s pulses from a sonifier (model 450, Branson Ultrasonics, Danbury, CT) (setting no. 7) using a microprobe (Branson Ultrasonics). This treatment completely disrupted the chloroplasts as observed under the microscope. The lysate was centrifuged for 5 min at 14,000*g* in an Eppendorf microcentrifuge (Brinkmann) and the supernatant was collected. The pellet was washed once and the wash was added to the first supernatant.

Mitochondria Isolation and Purification

Mitochondria were purified from 1.25 L of settled *D. innoxia* cells collected from cell-suspension cultures. The medium was washed from the cells by repeated suspension and centrifugation at 800*g* using a clinical centrifuge (International Equipment) with mitochondria extraction buffer (30 mM Hepes, pH 7.6, 3 mM DTT, 300 mM mannitol, 0.2% PVP-10, 1 mM EDTA, and 0.1% defatted BSA). Washed cells were suspended in a final volume of 2 L of extraction buffer. Cells were ruptured by passage through a milk homogenizer and filtered through cheesecloth and Miracloth. The filtrate was centrifuged at 5,000*g* for 2 min at 4°C. The supernatant was collected and centrifuged at 12,000*g* for 20 min. Pellets were suspended in mitochondria wash buffer (10 mM Hepes, pH 7.4 final, 300 mM mannitol, 1 mM EDTA, and 0.1% defatted BSA) and centrifuged at 1,500*g* for 5 min. The supernatant was collected and centrifuged at 12,000*g* for 10 min. Pellets were suspended in wash buffer and loaded onto 0 to 10% PVP Percoll (Pharmacia) gradients as described by Moore and Proudlove (1978). Loosely pelleted mitochondria were suspended in mitochondria wash buffer without BSA and centrifuged at 11,000*g* to pellet. This wash was repeated twice. Mitochondria were suspended in mitochondria lysis buffer (50 mM Tris, pH 7.5, 1 mM DTT, and 2 mM $MgCl_2$), sonicated for two 10-s bursts with a microprobe, and centrifuged at 4°C in a microfuge. The supernatant was collected and the pellet suspended in mitochondria lysis buffer containing 0.05% Triton X-100 (Sigma). Suspended pellets were centrifuged again. The two supernatants were pooled. The DTT was removed by filtration/concentration of the sample using a 30-kD exclusion filter (Amicon filtration membranes) prior to use in enzyme assays.

D. innoxia Cell-Culture Protoplasts

Two hundred milliliters of a wild-type *D. innoxia* suspension culture (where 120 mL was the settled cell volume) was used for protoplast isolation. An equal volume of filter-sterilized protoplasting solution (600 mM KCl, 2 mM NH_4NO_3, 3 mM $CaCl_2$, 1 mM KH_2PO_4, 1% [w/v] cellulase [Worthington Biochemicals, Freehold, NJ], 0.2% [w/v] pectolyase [Calbiochem], pH adjusted to 5.5 to 5.7 with KOH) was added to the cell culture and incubated for 45 min at 30°C with agitation at 150 rpm on a platform shaker incubator. The digested material was filtered through a sterile, 94-μm membrane, and the filtrate was centrifuged for 3 min in 50-mL Falcon tubes at 75*g* in a swinging-bucket rotor to concentrate the protoplasts. The loose pellet was washed once with 1:1 (v/v) culture medium/protoplasting solution (without the enzymes) at room temperature. Protoplasts were pelleted at 75*g* as above and washed twice at room temperature in transfer solution (0.4 M mannitol, 19 mM KNO_3, 1.25 mM KH_2PO_4, 3 mM $CaCl_2$, and 1.5 mM $MgSO_4$). After the second wash, protoplasts were suspended in lysis solution (0.3 M mannitol, 50 mM Tris, pH 7.6, and 2 mM $MgCl_2$) and counted using a hemocytometer. A portion of the protoplast preparation was disrupted in a sonicator as described above for chloroplasts and centri-

fuged at 12,000*g* in a microcentrifuge for 10 min at 4°C. The supernatant contained the total soluble proteins from cell-culture protoplasts. The remainder of the protoplasts were passed twice through a 20-μm nylon membrane (Spectra/mesh, Spectrum Lab Products, Houston, TX) attached to a 3-mL syringe using pressure to gently disrupt the protoplast membranes without disrupting the organelles. The filtrate was centrifuged at 500*g* in a swinging-bucket rotor for 10 min at 15°C to pellet the chloroplasts and membrane debris. The supernatant was collected and centrifuged at 12,000*g* in a microcentrifuge for 10 min at 4°C to pellet the mitochondria. The supernatant contained the cell-culture cytosolic protein.

D. innoxia Leaf Protoplasts

The leaf physiological state and the composition of enzymes and osmoticum in the protoplasting buffer were critical for obtaining high protoplast yields from *D. innoxia* leaves. Plants used as a source of protoplasts were grown from seed to the four- to five-leaf stage in pots of fine vermiculite, and were fertilized biweekly using a solution containing 190 mM KNO_3, 60 mM $CaCl_2$, 15 mM $MgSO_4$, and 25 mM KH_2PO_4 and the inorganic micronutrients of Murashige and Skoog (1962). Plants were illuminated by a pair of 75-W bulbs (one standard bulb and one wide-spectrum bulb) placed 3 feet above the pots. Twenty young (one-half to two-thirds expanded) leaves were used to generate protoplasts in a total volume of 1.2 L of enzyme digestion solution (20 mL solution per 8–10 cm^2 leaf area, which is about 0.3 g wet weight of tissue). Total leaf weight was 31 g. Leaves were collected, weighed, and rinsed in water. The undersides were scored with a soft brush and leaves were cut into 1-cm^2 pieces. Leaf pieces were gently vacuum-infiltrated for 1 min with a filter-sterilized enzyme-digestion solution (10 mM Mes, 0.3 M Suc, 4.75 mM KNO_3, 0.31 mM KH_2PO_4, 0.75 mM $CaCl_2$, 0.375 mM $MgSO_4$, 1.5% cellulase [Worthington], 0.05% pectolyase [Sigma], 0.5% macerase [Calbiochem], pH 5.6) and incubated at 27°C with shaking at 40 rpm for 2 h. Protoplasts were derived primarily from leaf palisade parenchyma cells. The digested material was passed through a sterile, 94-μm filter. Protoplasts in the filtrate were collected by centrifugation at 100*g* in a swinging-bucket rotor for 10 min at 15°C. The pelleted protoplasts were washed twice in protoplasting solution without enzyme. Protoplasts were collected, suspended in rinsing solution (0.4 M mannitol, 19 mM KNO_3, 1.25 mM KH2PO4, 3 mM $CaCl_2$, and 1.5 mM $MgSO_4$), and centrifuged as above. The pellets were suspended in a solution containing 0.3 M mannitol, 50 mM Tris, pH 7.6, and 2 mM $MgCl_2$ and counted using a hemocytometer. Protoplasts were lysed by forcing them three times through a 20-μm membrane attached to a 3-mL syringe, and the lysate was centrifuged to remove intact chloroplasts and mitochondria as described above. A portion of the intact protoplast preparation was sonicated and centrifuged as described for cell protoplasts and constituted the total leaf protoplast protein.

Organelle and protoplast extractions were repeated at least twice. Data presented are from one of the repeated extractions and are representative of characteristics observed in all repeated experiments.

Protein Determination

Protein concentration was determined spectrophotometrically by the method of Bradford (1976), using assay dye reagent (Bio-Rad) and BSA as the standard.

Enzyme Activity Assays

OASS

This activity was assayed by measuring the production of L-Cys as described previously (Kuske et al., 1994). The 1-mL reaction mixtures contained 100 mM Tris, 20 mM *O*-acetylserine, 0.2 or 1 mM Na_2S at a reaction pH of 7.6. *O*-Acetylserine and Na_2S were freshly prepared as 1-M and 100-mM stocks, respectively, in 100 mM Tris, pH 8.0, just prior to use. Reaction mixtures were preincubated at 34°C for 2 min, and reactions were initiated by the addition of 1 to 25 μL of protein sample. Each reaction was allowed to proceed at 34°C for exactly 10 min. Reactions were stopped by the addition of 200 μL of 1.5 M TCA. Precipitated protein was pelleted by centrifugation for 5 min in a microcentrifuge.

The amount of Cys present in the supernatant was determined as described by Gaitonde (1967). One milliliter of the supernatant was transferred to a glass tube and mixed well with an equal volume of acid ninhydrin reagent (250 mg of ninhydrin in 10 mL of glacial acetic acid:concentrated HCl [3:2, v/v]). The mixture was heated in a boiling-water bath for exactly 5 min, then cooled on ice. Two milliliters of cold 100% ethanol was added to each reaction and mixed well. The Cys concentration was determined spectrophotometrically by A_{546}. This assay is linear for L-Cys at concentrations between 0.01 and 0.5 μmol. The OASS activity assay was optimized with respect to pH, temperature, and concentration of each component, and was linear with respect to the amount of enzyme assayed.

A series of marker enzymes were used to determine the purity of isolated organelle preparations. Each assay was conducted at 25 to 27°C under conditions in which measured enzyme activity was linear with respect to the amount of sample enzyme protein added. Each assay was replicated three to four times for every protein sample.

Chloroplast Marker Enzymes

ADP-Glc pyrophosphorylase (EC 2.7.7.27) activity was measured by coupling the production of Glc-1-P to the reduction of NADP (Espada, 1966). The 1-mL reaction volumes contained 50 mM Tris buffer, pH 8.0, 5 mM $MgCl_2$, 2 mM DTT, 5 mM NaF, 1 mM sodium pyrophosphate, 1 mM ADP-Glc, 5 mM glycerate-3-phosphate, 10 μM Glc-1,6-bisP, 0.8 unit of phosphoglucomutase (EC 5.4.2.2), and 0.7 unit of Glc-6-P dehydrogenase (EC 1.1.1.49). Reactions were initiated by the addition of 500 μM NADP. NADP reduction was measured as increased A_{340} using a spectrophotometer (Lambda-Bio, Perkin-Elmer).

Glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$, phosphorylating, EC 1.2.1.13) activity was monitored by coupling the production of glyceraldehyde-3-phosphate to the oxidation of NADPH, measured as the change in A_{340}. Reactions were conducted in 1-mL volumes at pH 8.2, and contained 50 mM Hepes-NaOH, 1 mM $MgCl_2$, 4 mM EDTA, 5 mM DTT, 1 mM ATP, 0.2 mM NADPH, 10 units of phosphoglycerate kinase (EC 2.7.2.3), and 10 units of triose-phosphate isomerase (EC 5.3.1.1) (Lunn et al., 1990). Ten microliters of protein fraction was incubated with the above reaction mixture for 5 min (Jackson et al., 1979). The reaction was initiated by the addition of 10 mM 3-phosphoglycerate.

Mitochondrial Marker Enzyme

Citrate synthase (EC 4.1.3.7) activity was measured in 1-mL reactions that contained 50 mM Hepes-KOH, pH 7.6, 0.25 mM $MgCl_2$, 1 mM oxaloacetate, 0.2 mM acetyl CoA, and 1.5 mM 5,5′-dithiobis-(2-nitrobenzoic acid). Reactions measured the release of CoA, which was followed by measuring the change in A_{412} (Cooper and Beevers, 1969).

Peroxizome Marker Enzyme

Hydroxypyruvate reductase (EC 1.1.1.81) activity was measured as the decrease in A_{340} due to oxidation of NADH in reaction mixtures that contained 50 mM Mes-NaOH, pH 6.4, 0.2 mM NADH, and 1 mM hydroxypyruvate. Protein samples were incubated in the buffer NADH mixture for 2 min to measure endogenous rates. Reactions were then initiated by the addition of hydroxypyruvate (Tolbert et al., 1970).

Cytosolic Marker Enzyme

Activity of pyrophosphate:Fru-6-P-1-phosphotransferase (EC 2.7.1.90) was measured by coupling the Pi-dependent conversion of Fru-6-P to Fru-1,6-bisP to oxidation of NADH and measuring the change in A_{340} (Edwards et al., 1984). Reactions contained 100 mM Hepes-NaOH (pH 7.8), 1 mM $MgCl_2$, 5 mM Fru-6-P, 0.15 mM NADH, 1 unit of glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), 10 units of triose-phosphate isomerase (EC 5.3.1.1), and 0.4 unit of aldolase (EC 4.1.2.13). Protein samples were preincubated for 2 min in Hepes buffer, pH 7.8, with 2 μM Fru-2,6-bisP and 5 mM NaF (Rolland et al., 1992), then added to the rest of the reaction mixture (final reaction volume 1 mL). Reactions were initiated by the addition of 1 mM sodium pyrophosphate.

Anion-Exchange HPLC of Protein Extracts

Protein samples were applied to a DEAE-5-PW column (Bio-Gel SEC, Bio-Rad; 75 × 7.5 mm) in 10 mM Tris, pH 8.1, and eluted in a 60-mL linear gradient of 0 to 300 mM NaCl in 10 mM Tris, pH 8.1, to separate the three *D. innoxia* OASS forms (Kuske et al., 1994). Protein eluting from the column was continuously monitored by A_{280} (data not shown). One-milliliter fractions were collected and assayed for OASS activity.

RESULTS

Organelle Isolation from *D. innoxia*

Chloroplasts of high purity were readily isolated from photosynthetic *D. innoxia* leaf tissue (Table I); however, it proved difficult to obtain good chloroplast yields from cell cultures. Although plastids were abundant in cell cultures, they were full of starch crystals and ruptured very easily. Cell cultures could not regain photosynthetic ability when exposed to light, or when the carbohydrate source in the medium was reduced, eliminated, or changed to an alternative carbohydrate such as mannitol (data not shown).

Mitochondria were most easily isolated from cell cultures. However, mitochondrial yields from *D. innoxia* leaves were very low and, even after purification, were contaminated with chloroplasts and peroxizomes (as indicated by marker enzyme activities; data not shown). The mitochondrial preparations from cell cultures were greatly enriched for this organelle, but also contained some cytosolic marker enzyme activity (Table I).

Table I. *Distribution of OASS and marker enzyme activity in D. innoxia leaves and cell suspension cultures*

Presence of OASS and marker enzyme activity in protein preparations from the chloroplasts, mitochondria, cytosol, leaves, and cell cultures of *D. innoxia*. Values in the table are the average of three to four replicate assays followed by the SE in parentheses. Marker enzymes were AGP (ADP glucose pyrophosphorylase, EC 2.7.7.27), G3PD (glyceraldehyde-3-phosphate dehydrogenase, NADP, phosphorylating, EC 1.2.1.13), CS (citrate synthase, EC 4.1.3.7), HR (hydroxypyruvate reductase, EC 1.1.1.81), and PPi:FPP (pyrophosphate:fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.90). Where enzyme activity was not detected in the G3PD assay, no product formation occurred over 10 min at several protein concentrations. The limit of detection for this assay was about 0.4 nmol/min.

Cell Fraction	OASS	Chloroplast		Mitochondria	Peroxizome	Cytosol PPi:
		AGP	G3PD	CS	HR	FPP
	nmol product formed min^{-1} mg^{-1} protein					
Chloroplasts (leaves)	5,155 (81)	149.0 (3.4)	281.0 (6.6)	23.6 (2.0)	Not tested	10.2 (0.8)
Mitochondria (cells)	11,598 (25)	13.2 (8.7)	Not detected	2558 (86)	342 (39)	102 (14.3)
Cytosolic (leaf protoplasts)	847 (55)	67.0 (5.2)	11.6 (0.8)	18.5 (0.2)	1166 (17)	67.3 (6.5)
Cytosolic (cell protoplasts)	318 (4.3)	10.5 (0.6)	2.2 (0.0)	19.0 (0.3)	0.3 (0.2)	529 (3.8)
Leaves	1,183 (27)	83.1 (0.4)	47.9 (1.7)	12.5 (0.2)	2170 (131)	12.7 (0.7)
Cell culture	5,103 (103)	24.3 (0.6)	Not detected	638 (6.7)	2305 (82)	221 (7.7)

Cellular Location of Three OASS Isoenzymes

Three isoenzymes of OASS that differ in size and subunit structure can be resolved using anion-exchange HPLC on DEAE-5-PW. These were designated isoenzymes A, B, and C in the order in which they were identified during purification from plant cells (Kuske et al., 1994). Isoenzyme A elutes at about 120 mM NaCl, followed by isoenzyme C at about 140 mM NaCl, and isoenzyme B at about 245 mM NaCl. The in vitro specific activity of the three OASS isoforms is the same, so we were able to separate and compare the relative amount of each isoenzyme in the organelle and protoplast preparations by measuring the activity that eluted at different times from the column.

D. innoxia leaves, roots, and heterotrophic cell cultures are in different physiological states, so their requirements for Cys are likely to differ. The same three OASS isoenzyme forms that were originally identified in cell-suspension cultures were present in leaves and root tissues of *D. innoxia* plants. However, the relative amount of each isoenzyme present in green leaves, roots, and nongreen cell cultures differed greatly (Fig. 1).

Isoenzyme B was the predominant form in green leaves, constituting 51% of the total activity in this tissue; it was also the major isoenzyme present in purified chloroplasts from *D. innoxia* leaf tissue (83%), but was present only as a minor form in cell cultures, roots, and purified mitochondria. The abundance of this isoenzyme in photosynthetic leaf tissue and in chloroplasts isolated from leaves (Fig. 1) and its absence in preparations from nonphotosynthetic roots and cell cultures indicate that it is the chloroplast form of OASS.

Isoenzyme A was the most abundant form in the cytosolic protein fractions obtained from leaf (65%) and cell culture (54%) protoplasts (Fig. 2), suggesting that it is the predominant cytosolic form of OASS. This form was also the most predominant in nonphotosynthetic roots (89%) and heterotrophic cell cultures (58%) (Fig. 2).

Cell-suspension cultures contained substantial amounts of both isoenzymes A and C (Fig. 1), with isoenzyme A the predominant form. The proportion of isoenzyme C relative to isoenzyme A was increased in repeated mitochondrial preparations, suggesting that it may be a mitochondrial form of this enzyme. However, the isolated mitochondria could not be purified entirely from contaminating cytosolic proteins (as indicated by marker enzymes; Table I). Our data indicate that isoenzyme A is predominant in the cytosol and that isoenzyme C is predominant in the mitochondria, but not that their presence in either location is mutually exclusive.

D. innoxia cell cultures are capable of tolerating and accumulating high concentrations of cadmium and other metals (Jackson et al., 1989, 1992; Jackson and Kuske, 1993). The relative abundance of OASS isoforms in $Cd300^R$ in the presence and absence of 300 μM cadmium, in the wild-type culture (which is sensitive to cadmium), and in $Cd300^S$ originally derived from the cadmium-tolerant cell line, were compared to determine if the total amount of OASS activity or the amount of any of the individual isoforms

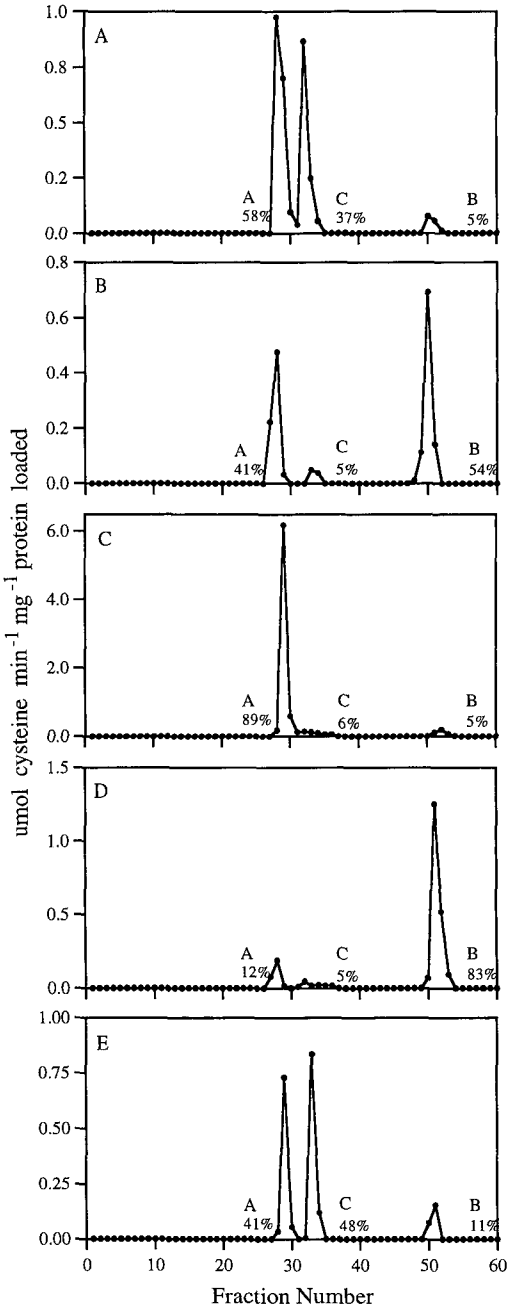

Figure 1. The presence and relative abundance of three OASS isoenzymes in *D. innoxia* cell culture (A), green leaves (B), roots (C), leaf chloroplasts (D), and cell-culture mitochondria (E). OASS isoenzymes were separated by anion-exchange chromatography on DEAE-5-PW. Samples were applied in 10 mM Tris, pH 8.1, and eluted in a 60-mL linear gradient of 0 to 300 mM NaCl in 10 mM Tris, pH 8.1, where isoenzymes A, C, and B elute at 120, 140, and 245 mM NaCl, respectively. The amount of protein loaded on the column was: cell culture, 4 mg; leaves, 3 mg; roots, 1 mg; chloroplasts, 0.85 mg; and mitochondria, 0.2 mg. One-milliliter fractions were collected and assayed for OASS activity as described in the text. Values below each peak identification are the percentage of total OASS activity in that peak.

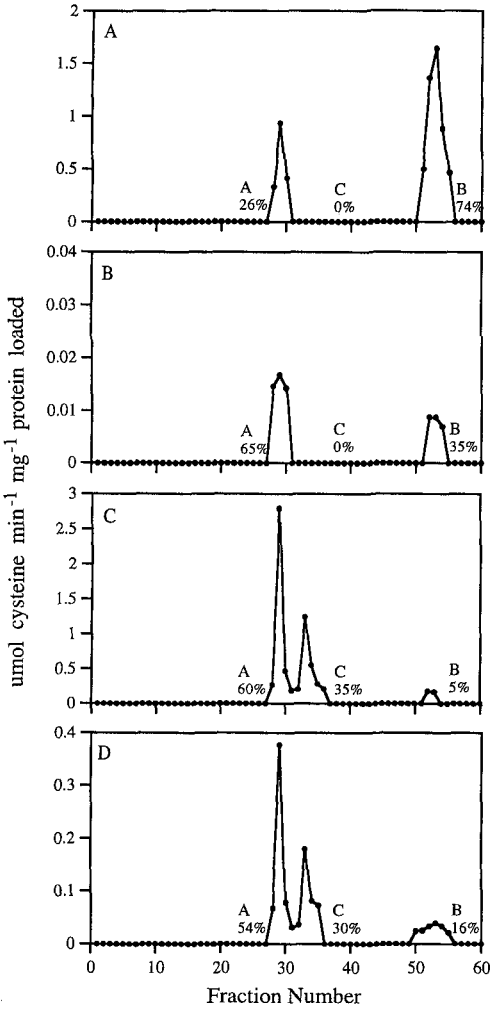

Figure 2. The presence and relative abundance of three OASS isoenzymes in total protein (A and C) and cytosolic proteins (B and D) from *D. innoxia* leaf and cell-culture protoplasts, respectively. OASS isoenzymes were separated by anion-exchange chromatography on DEAE-5-PW as described in Figure 1. The amount of protein loaded on the column was 0.6 mg (A), 6.5 mg (B), 1 mg (C), and 9 mg (D). One-milliliter fractions were collected and assayed for OASS activity as described in the text. Values below each peak identification are the percentage of total OASS activity in that peak.

differed among the cell cultures. In the absence of cadmium, the total OASS activity present in the cadmium-tolerant cell line (1.51 μmol Cys min^{-1} mg^{-1} protein) was 1.8 times as high as in WDI (0.84 μmol Cys min^{-1} mg^{-1} protein), and was about 2.9 times as high as in the highly cadmium-sensitive cell line (0.52 μmol Cys min^{-1} mg^{-1} protein). In the presence of 300 μM cadmium, the total amount of OASS activity in the cadmium-tolerant cell line after 24 h of exposure decreased to one-half the original value (0.82 μmol Cys min^{-1} mg^{-1} protein). The OASS profiles from anion-exchange chromatography on DEAE-5-PW were similar for all cell lines and cadmium exposures (see Fig. 1A for example).

DISCUSSION

Reduced sulfur in the form of Cys is needed for many different functions in plant cells (Rennenberg, 1984; Rennenberg et al., 1990; Schmidt and Jäger, 1992). It is essential for normal plant metabolism, as the carrier of reduced sulfide for synthesis of Met, certain co-factors, and membrane components, and as an essential amino acid for protein synthesis. It is also essential as a precursor for glutathione and other stress-response metabolites. Sulfur uptake and cellular needs for Cys vary during plant growth and differentiation (see, for example, von Arb and Brunold 1986; Ghisi et al., 1993). They also vary as the plant responds to changes in its environment, including sulfate availability, light, and the presence of abiotic and biotic factors producing oxidative stress (Smith, 1972; Saccomani et al., 1984; Nussbaum et al., 1988; Delhaize et al., 1989; Rauser et al., 1991; Hell et al., 1994). The Cys pool in plant cells is tightly regulated, and has been found to remain relatively constant, even though cellular sulfur content and amounts of other sulfur-containing metabolites may fluctuate greatly during plant growth and response to stress. The need for three cellular sites of Cys synthesis and the predominance of different isoforms in different tissues and cell types suggest that the OASS isoforms produce Cys that is used for different functions in the plant cell. The three isoforms are probably differentially regulated in response to different cellular needs and environmental conditions.

Multiple forms of OASS have been identified in several plants using anion-exchange chromatography (Ikegami et al., 1988; Lunn et al., 1990; Rolland et al., 1992; Kuske et al., 1994). The three forms of the enzyme from *D. innoxia* are located in the chloroplast, mitochondria, and cytosol, with a different form predominant in each location. In addition, the amount of each form varies dramatically in different cell types and plant tissues. These isoenzymes have similar in vitro catalytic ability but different physical properties, suggesting that they are encoded by different genes or are processed differently by the cell to produce Cys as a precursor for different aspects of sulfur metabolism in the organelles and cytosol.

OASS activity is required as the final step in assimilation of inorganic sulfide in plants. Chloroplasts are considered to be the primary site for reductive sulfate assimilation (Brunold and Suter, 1982; Lunn et al., 1990), since they contain all of the enzymes necessary for this function and since isolated plant chloroplasts are able to reduce sulfate (Schmidt, 1986). OASS activity has been localized to the chloroplasts of spinach (Lunn et al., 1990) and the plastids of cauliflower (Rolland et al., 1992). *D. innoxia* photosynthetic leaf cells, nonphotosynthetic roots, and heterotrophic suspension-cell cultures differ greatly in their amounts of isoenzyme B, the chloroplast form of OASS. The chloroplast form (isoenzyme B) is the predominant form in photosynthetically active leaf cells, but is a minor form in the roots and in nongreen cell-suspension cultures. The abundance of the chloroplast OASS in photosynthetic cells reflects the need for Cys in sulfate assimilation and protein synthesis, and suggests that the chloroplasts are the primary site for both functions in *D. innoxia* leaf tissue.

In contrast to the photosynthetic leaves, the predominant OASS form in *D. innoxia* roots is the cytosolic form (isoenzyme A). This form constitutes about 89% of the total activity in this tissue, with only minor amounts of the other two forms present. The difference in predominant OASS forms between the leaves (chloroplast form) and roots (cytosolic form) suggests that these two tissues have different requirements for cellular Cys. Whether this is determined by the presence of photosynthetically active plastids in the tissue is unclear. The OASS composition in nonphotosynthetic cauliflower buds is similar to that found in *D. innoxia* photosynthetic leaf tissue. In the cauliflower buds, a plastid form of OASS was the most abundant form (40–45% of activity), and two other forms represented significant amounts of activity in this tissue (Rolland et al., 1992).

The mitochondrial and cytosolic forms of OASS are abundant in suspension-cell cultures. This composition represents a significant decrease in the chloroplast OASS compared with green leaves, and an increase in mitochondrial OASS compared with either leaf or root tissues. These cell cultures multiply very rapidly, doubling in number every 24 h, and mitochondria are abundant. It is possible that in the absence of photosynthetically active plastids sulfate assimilation occurs primarily in the mitochondria. The presence of other enzymes of sulfate assimilation in the mitochondria support this possibility. ATP sulfurylase activity is present in pea mitochondria (Lunn et al., 1990), and Ser transacetylase activity has been localized in the mitochondria of bean (Smith, 1972). In the alga *Euglena gracilis* the enzymes of reductive sulfate assimilation are located in the mitochondria of light- and dark-grown normal cells and in a mutant cell line that lacks plastids (Brunold and Schiff, 1976; Saidha et al., 1988). Mitochondrial forms of OASS have also been reported from pea and cauliflower plants (Lunn et al., 1990; Rolland et al., 1992). These results suggests that the mitochondria may be a second site for sulfate assimilation, even in photosynthetic plant tissues. In the absence of photosynthetically active plastids in *D. innoxia* cell cultures, this function may be assumed primarily by the mitochondria.

The *D. innoxia* cell-suspension cultures contain abundant plastids, but they are not photosynthetically active. This change appears to be permanent, since photosynthetic activity could not be induced by light or by adjustment of the media to reduce, change, or eliminate the carbon source. The *D. innoxia* cell cultures and plant tissues differed in both amount and composition of total soluble protein (data not shown). The cell cultures contained far less total protein than the leaves, presumably because they are deficient in the enzymes needed for photosynthesis. They appear to be deficient in other enzymes as well. Glyceraldehyde-3-phosphate dehydrogenase activity, although abundant in plant tissues, could barely be detected in the suspension-cell cultures (Table I). Cell cultures are useful models for some aspects of normal and stress-related plant metabolism; however, the differences in predominant OASS forms between cell cultures and plant tissues indicate that they may have altered physiology regarding Cys synthesis, which could dramatically affect the processes of protein synthesis, sulfate assimilation, and glutathione-mediated stress response.

Ser acetyltransferase (EC 2.3.1.30) produces the *O*-acetylserine used as substrate by OASS. In bacteria about 5% of the OASS is found associated with Ser acetyltransferase as a high-molecular-weight complex called Cys synthetase, which contains two molecules of the former enzyme and one molecule of the latter enzyme (Kredich and Tompkins, 1966; Kredich et al., 1969). Droux et al. (1992) identified a similar enzyme complex in spinach chloroplasts. However, we found no evidence for a similar high-molecular-weight complex in *D. innoxia* cell cultures (enriched for the cytosolic and mitochondrial forms of OASS; Kuske et al., 1994). In both bacteria and plants, most of the OASS is present as a free enzyme.

Although both Ser acetyltransferase and OASS are present in the cytosol and organelles, the relative amount of each enzyme in the different cellular compartments differs greatly. We found that most of the *D. innoxia* leaf OASS was in the chloroplasts, whereas only 10% of the pea leaf Ser acetyltransferase is in this organelle (Ruffet et al., 1995). In pea leaves most (76%) of Ser acetyltransferase is in the mitochondria. In pea chloroplasts the amount of Ser acetyltransferase is very low relative to the amount of OASS (1:345), a ratio that allows the maximum production of Cys by the two enzymes in this organelle (Ruffet et al., 1994). Presumably, activity of these two enzymes must be coordinated in the cell for efficient production of Cys. However, the metabolic significance of their vastly different abundance in various organelles and the presence of a Ser acetyltransferase/*O*-acetyltransferase enzyme complex in plants remains unclear.

Plant response to a variety of abiotic and biotic stresses is mediated by glutathione. Glutathione functions as a reducing agent to protect against oxidative damage, detoxifying drugs, herbicides, and other xenobiotics by conjugation, and is a precursor in the metal-induced biosynthesis of phytochelatins [$(\gamma\text{-EC})_n$Gs], the heavy-metal-binding polypeptides that function to bind accumulated toxic metals (Rauser, 1990; Steffens, 1990; Jackson and Kuske, 1993; Robinson et al., 1993). Cys is a precursor for glutathione and the glutathione derivatives that respond to plant stress. One important function of the OASS isoenzymes is to produce Cys for glutathione-mediated response to plant stress. Such stress responses are often rapid and dramatic (Delhaize et al., 1989). An effective glutathione-mediated stress response will thus require considerable amounts of cellular Cys in a short time.

Uptake of inorganic sulfate is increased in plants exposed to cadmium (Nussbaum et al., 1988). Cadmium exposure also results in rapid depletion of the glutathione pool as the amounts of metal-binding phytochelatins increase (Delhaize et al., 1989; Rauser et al., 1991). OASS activity is clearly required to establish and maintain sufficient cellular Cys pools required for glutathione synthesis. Certain cell lines of *D. innoxia* tolerate high cadmium concentrations in the growth medium. To investigate the potential role of OASS activity and the three isoenzymes in the cadmium-stress response we determined the total amount of OASS activity and the relative amount of each

isoenzyme form in $Cd300^R$ and $Cd300^S$. The OASS composition of cadmium-tolerant cells in the presence and absence of cadmium was compared to determine whether the *D. innoxia* isoenzymes respond differently to abiotic stress requiring Cys. $Cd300^R$ contained higher amounts of constitutive OASS activity than the wild-type or cadmium-hypersensitive cell lines. All three cell lines are able to synthesize metal-binding polypeptides in response to cadmium. However, only the tolerant cells survive the first 24 h after cadmium exposure (Delhaize et al., 1989). The higher constitutive amounts of OASS isoenzymes present in the cadmium-tolerant cell line may allow faster cell response and greater glutathione synthesis in the first critical hours after cadmium exposure, which could allow the cells to survive these high cadmium concentrations. After a 24-h exposure to cadmium the total OASS activity in the cadmium-tolerant cell line was reduced, and relative amounts of all three isoenzyme forms remained the same, indicating that with the higher constitutive level of OASS activity, the Cys pool may have been adequate for response to cadmium without additional synthesis of OASS, or that there may be an OASS response at an earlier time following cadmium exposure.

D. innoxia cells have the ability to synthesize Cys in three cellular compartments to provide reduced sulfur for many cellular functions. We have demonstrated that different plant tissues and plant cells in different physiological states contain different amounts of these compartmentalized enzymes, reflecting their different needs for Cys. Whether Cys is provided within different cellular compartments because it is potentially toxic to plant cells (Giovanelli, 1987), because it does not readily move across organelle membranes (Lunn et al., 1990), or because the local Cys pool regulates the use of reduced sulfur for different cellular processes is unknown. The three differentially compartmentalized OASS isoenzymes are likely to have different functions in sulfur metabolism in the plant cell, and will be differentially regulated by different environmental conditions and developmental needs.

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