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Localization of γ-Glutamylcysteine Synthetase and Glutathione Synthetase Activity in Maize Seedlings[1]

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Fresh weight, protein, cysteine, γ-glutamylcysteine, glutathione, and the extractable activity of the enzymes of glutathione biosynthesis, γ-glutamylcysteine synthetase (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3), were measured in roots, scutella, endosperms, and shoots of 3-, 7-, and 11-d-old maize (*Zea mays* L. cv LG 9) seedlings. In 3-d-old seedlings, the scutella represented 14% of the seedling fresh weight, containing 43% of total protein and 63 and 55% of the activity of γ-glutamylcysteine synthetase and glutathione synthetase, respectively; in 11-d-old seedlings, the corresponding values were 4.5% for fresh weight, 8.0% for protein content, and 14 and 20% for the enzyme activities. The highest concentrations of thiols were found for cysteine (0.27 mM) in the roots, for glutathione (4.4 mM) in the shoots, and for γ-glutamylcysteine (13 μM) in the scutella of 3-d-old seedlings. The enzyme activities of roots were localized in subcellular fractions after sucrose density gradient centrifugation. Nearly half of the γ-glutamylcysteine synthetase activity was detected in the root proplastids of 4-d-old seedlings, whereas <10% of the glutathione synthetase activity was localized in this organelle. Our results demonstrate the importance of scutella in glutathione synthesis in the early stage of seedling development. Unlike chloroplasts, root plastids show only a small proportion of glutathione synthetase activity.

Although GSH is one of the major low mol wt compounds, with a variety of physiological functions in plants (Rennenberg, 1987), comparably little information is available about the enzymes catalyzing GSH synthesis in plant tissues. Similar to what has been described for animals (Meister, 1985; Seelig and Meister, 1985) and microorganisms (Meister and Anderson, 1983; Dennda and Kula, 1986), in which the corresponding enzymes have been studied intensively, GSH synthesis in plants also takes place in two steps (Hell and Bergmann, 1990). In the first step, catalyzed by γEC synthetase (EC 6.3.2.2), the dipeptide γEC is produced from L-glutamate and L-Cys in an ATP-dependent reaction (Lancaster et al., 1989; Steffens and Williams, 1989; Hell and Bergmann, 1990; Rüegsegger and Brunold, 1992). In the second step, Gly is added to the C-terminal site of the dipeptide to yield GSH; this reaction is catalyzed by GSH synthetase (EC 6.3.2.3) and depends also on ATP (Law and Halliwell, 1986; Klapheck et al., 1987; Macnicol, 1987; Hell and Bergmann, 1988; Rüegsegger et al., 1990). Corresponding enzyme activities have been measured in extracts from leaves (Law and Halliwell, 1986; Klapheck et al., 1987; Macnicol, 1987; Hell and Bergmann, 1990) and roots (Rüegsegger at al., 1990; Rüegsegger and Brunold, 1992). The scutellum is presumed to play an important role in GSH synthesis of maize seedlings (Rauser et al., 1991), but data concerning the GSH-synthesizing enzymes from this organ are lacking.

Pea (*Pisum sativum*) roots have been shown to contain the first three enzymes of the sulfate assimilatory pathway (ATP-sulfurylase, APSSTase, and sulfite reductase) localized exclusively or almost exclusively in the proplastids, whereas *O*-acetyl-L-Ser sulfhydrylase was detected predominantly in the cytoplasm (Brunold and Suter, 1989). In addition to the activities of γEC synthetase and GSH synthetase measured in maize root extracts, isolated maize roots incorporated radiolabel from [^{35}S]sulfate into Cys, γEC, and GSH, showing the capacity for both sulfate assimilation and GSH synthesis in vivo (Rüegsegger and Brunold, 1992). Special interest attaches to the sulfur metabolism of the root, because this plant organ can be seriously affected by heavy metals and by the action of a variety of herbicides, these xenobiotics being detoxified via mechanisms requiring GSH (Grill et al., 1989; Komives and Dutka, 1989; Rauser, 1990; Steffens, 1990). No information is available so far about the subcellular localization of γEC synthetase and GSH synthetase in root tissue. γEC synthetase was localized "very predominantly" in the chloroplasts obtained from onion mesophyll protoplasts (Lancaster et al., 1989) and to the extent of 72 and 61% in the chloroplasts from *Pisum sativum* and *Spinacia oleracea*, respectively (Hell and Bergmann, 1990). For GSH synthetase, 24 to 69% of the total activity from leaf cells was assigned to the chloroplasts, depending on the plant material used for chloroplast isolation (Klapheck et al., 1987; Hell and Bergmann, 1988; Hell and Bergmann, 1990).

In the present paper, we report the distribution of γEC synthetase and GSH synthetase activity in maize seedlings, including scutellum and endosperm, and the distribution and concentrations of Cys, γEC, and GSH in the different organs. In addition, the subcellular localization of γEC synthetase and GSH synthetase was examined in the root.

MATERIALS AND METHODS

Plant Material

Maize kernels (*Zea mays* L. cv LG 9, Limagrain, Ennezat, France) were soaked for 1 d in aerated water at room tem-

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Abbreviations: APS, adenosine 5′-phosphosulfate; APSSTase, adenosine 5′-phosphosulfate sulfotransferase; γEC, γ-glutamylcysteine.

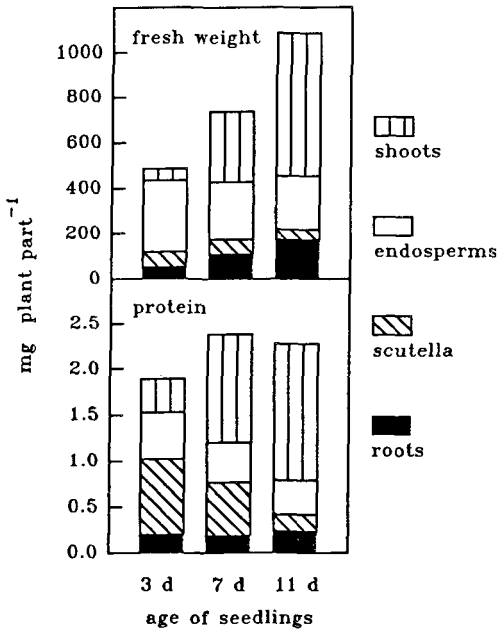

Figure 1. Fresh weight and protein content of maize seedling roots, scutella, endosperms, and shoots (including mesocotyl). Mean values of at least 20 measurements combined from four independent experiments are presented for fresh weight. The protein contents are derived from the protein measured in the desalted extracts used for enzyme activity determinations in four independent experiments.

perature and germinated between several layers of damp paper in the dark at 24 to 26°C for 2 d; 16 seedlings were placed in pots with 320 mL of nutrient solution (Nussbaum et al., 1988). The plants were cultivated in continuous light (70 μE m^{-2} s^{-1}, provided by three fluorescent TL 40 W/34 tubes from Philips and one L 36/W 77 Fluora from Osram) at 24 to 26°C and 60 to 65% RH. For sucrose density gradient experiments, the kernels were transferred after imbibition into an aluminum box containing 1.8 L of one-quarter-strength nutrient solution (Nussbaum et al., 1988) and kept for 3 d in the dark at 24 to 26°C on a steel grid in contact with the nutrient solution, which was aerated at a rate of 50 L/h.

Preparation of Extracts

Root systems were rinsed liberally with tap water. Roots and shoots were cut off with a razor blade as close as possible to the scutellum. Scutella were picked out with a scalpel. Roots, shoots, or scutella of at least three plants were ground in an ice-cooled glass homogenizer. Five or six endosperms were ground (while ice cooled) with a Polytron homogenizer (Kinematica, Littau, Switzerland) for 10 s at 6,000 rpm, twice for 10 s at 12,000 rpm, and for 10 s at 20,000 rpm. The homogenates were made cell free by filtration through one layer of 100% viscose fleece (Milette, Migros, Switzerland). For the thiol measurement, endosperms and scutella were extracted with 5 and 20 parts (w/v), respectively, of 0.1 N HCl containing 1 mM Na_2-EDTA. For roots and shoots, the extraction ratio was varied from 1:20 for 3-d-old plants to 1:10 for 7-d-old plants and 1:7 (w/v) for 11-d-old plants. The extracts for the measurement of γEC synthetase activity were prepared by grinding plant material in 0.1 M Tris-HCl containing 5 mM EDTA (pH 8.0). For the GSH synthetase assay, plant material was extracted in 0.1 M Tris-HCl containing 10 mM $MgCl_2$ and 1 mM EDTA (pH 7.5). For the enzyme measurements, the extraction ratio was 1:5 or 1:10 (w/v) depending on the different protein contents of the plant material. After the desalting step, the extracts contained 0.2 to 1.2 mg of protein per mL.

Determination of Cys, γEC, and GSH

Thiols were separated and quantified by reverse-phase HPLC after reduction with $NaBH_4$ and fluorescent labeling with monobromobimane (Newton et al., 1981; Schupp and Rennenberg, 1988) as previously described (Rüegsegger and Brunold, 1992).

Enzyme Assays

The activity of γEC synthetase was determined in extracts according to the method of Rüegsegger and Brunold (1992) by the quantification of the reaction product after reverse-phase HPLC separation of its monobromobimane derivative (Hell and Bergmann, 1990). For sucrose density gradient fractions, desalting was omitted and a shorter incubation time (30 min) and a higher concentration of DTE (1 mM) in the assays were used than for extracts. GSH synthetase activity was measured in extracts as previously reported (Klapheck et al., 1987; Rüegsegger and Brunold, 1992) using the same principle as mentioned for γEC synthetase. For sucrose den-

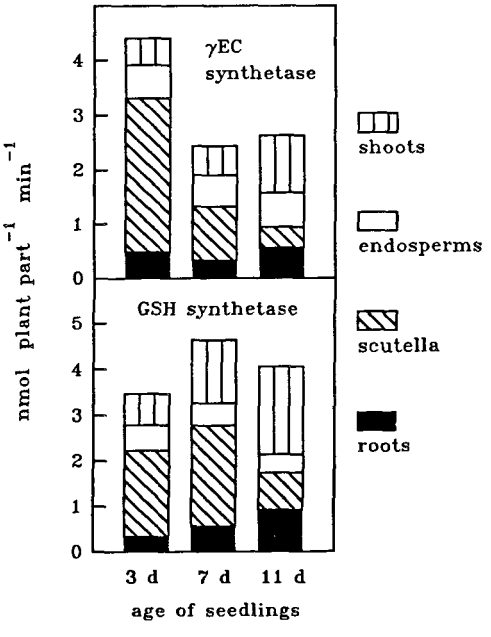

Figure 2. Extractable activity of γEC synthetase and GSH synthetase of maize seedling roots, scutella, endosperms, and shoots (including mesocotyl). Mean values of two independent experiments are presented. The mean difference of the parallel single values from their average was 10.1%.

Table I. *Concentrations of Cys, γEC, and GSH and extractable activity of γEC synthetase (γEC-S) and GSH synthetase (GSH-S) in roots, scutella, endosperms, and shoots (including mesocotyl) of 3-, 7-, and 11-d-old maize seedlings*

Mean values of two independent experiments are presented.

	Roots			Scutella			Endosperms			Shoots		
	3 d	7 d	11 d	3 d	7 d	11 d	3 d	7 d	11 d	3 d	7 d	11 d
	nmol g^{-1} fresh wt											
Thiols												
Cys	272	13.7	11.7	127	60.2	82.9	37.1	54.7	42.9	52.6	10.4	7.11
γEC	7.41	1.85	1.57	13.0	8.37	10.0	5.01	3.78	2.11	5.97	2.54	1.57
GSH	1860	492	312	1760	4350	3390	42.7	61.1	54.3	4360	616	396
	nmol g^{-1} fresh wt min^{-1}											
Activity												
γEC-S	8.76	3.00	3.55	41.1	15.2	7.22	1.96	2.30	2.75	8.90	1.72	1.80
GSH-S	6.64	4.96	5.05	27.0	36.1	17.9	1.73	1.89	1.67	13.4	4.33	2.81

sity gradient fractions, desalting was omitted and a shorter incubation time (45 min) was used than for extracts. The activity of ATP-sulfurylase was measured in the back-reaction by determining the ATP formed from PPi and APS using a luciferin-luciferase system (Schmutz, 1990). The activity of APSSTase was measured by the formation of [^{35}S]sulfite assayed as acid-volatile radioactivity from [^{35}S]APS in the presence of DTE (Brunold and Suter, 1990). For the measurement of enzyme activities in sucrose gradient fractions, all enzyme assays were adjusted to 0.01% (w/v) of the detergent Brij 58 (polyoxyethylene 20 cetyl ether) to break up intact organelles.

Sucrose Density Gradient Centrifugation

Subcellular fractions of 2- to 3-cm root tips were prepared using sucrose density gradients according to the method of Emes and Fowler (1979), but the 1 mM GSH present in the extraction medium (Emes and Fowler, 1979) was replaced by 0.5 mM DTE to avoid interferences with the measurement of GSH-synthesizing enzymes. Centrifugation was for 30 min at 19,000 rpm and 4°C using a Kontron (Munich, Germany) TST 28.38 rotor (Brunold and Suter, 1989). Fractions of 1.6 mL were collected. Sucrose concentrations were determined by measuring the refractive index with a Zeiss (Oberkochen, Germany) refractometer.

Protein Determination

The protein content of the extracts was measured according to the method of Bradford (1976) with BSA as the standard.

Chemicals

Monobromobimane was obtained from Calbiochem (La Jolla, CA), γEC from Nacalai Tesque (Kyoto, Japan), and Brij 58 was from Serva (Heidelberg, Germany). [^{35}S]APS was prepared according to the method of Li and Schiff (1991), using APS from Sigma (St. Louis, MO) and [^{35}S]sulfate from the Radiochemical Centre (Amersham, UK). All other chemicals were purchased from Fluka (Buchs, Switzerland).

RESULTS

The 2.2-fold increase in the fresh weight from 3- to 11-d-old maize seedlings was caused primarily by shoot growth and to a smaller extent by root growth (Fig. 1) with a gain in fresh weight of 578 mg per shoot (12.3-fold increase) and 118 mg per root system (3.2-fold increase). The shoot was the only plant part with a considerable gain in protein (1.1 mg, 4.1-fold) during this period, whereas scutella lost 0.64 mg (75%) of their protein (Fig. 1).

In 3-d-old seedlings, the extractable activities of both γEC synthetase and GSH synthetase (Fig. 2) were present in scutella in a proportion far larger than the fresh weight and even larger than the protein proportion of this organ. Whereas the activity of γEC synthetase in scutella decreased rapidly and continuously during seedling development, the activity of GSH synthetase in this organ increased between days 3 and 7 and decreased to a low level on day 11. As the seedlings developed, the shoots became the organ with the largest proportion of γEC synthetase and GSH synthetase activity (Fig. 2), but the increase in activity was clearly smaller than the increase in fresh weight and protein, leading to activity losses on a fresh weight basis of about 80% (Table I).

It is surprising that the roots of 3-d-old seedlings contained more Cys, γEC, and GSH than those of 7- or 11-d-old plants, showing the largest difference for Cys, which decreased from 13.7 nmol per root in 3-d-old seedlings to 2.0 nmol per root in 11-d-old seedlings (Fig. 3). Because the fresh weight of the roots increased 3.2-fold during this period, an even larger effect was observed on a fresh weight basis: the concentration of Cys decreased 23-fold (Table I). A considerable amount of Cys and only a very small amount of GSH, both without substantial variations during seedling development, were found in the endosperms (Fig. 3). The extraordinary ratio of approximately 1:1 of the amounts as well as the concentrations (Table I) of Cys compared to GSH is predominantly the result of the very low GSH concentration (42–61 μM) detected in endosperms. Millimolar concentrations of GSH were measured in roots (1.9 mM), shoots (4.4 mM), and scutella (1.8 mM) of 3-d-old seedlings (Table I). Whereas in roots and shoots

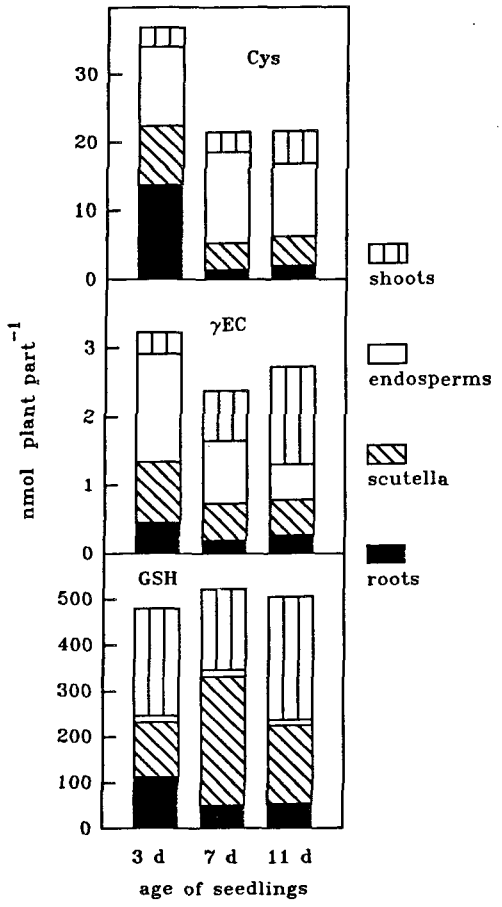

Figure 3. Contents of Cys, γEC, and GSH in maize seedling roots, scutella, endosperms, and shoots (including mesocotyl). Mean values of two independent experiments are presented. The mean difference of the parallel single values from their average was 7.2%.

GSH was diluted by growth and no net gain of this compound was observed, an accumulation of GSH up to 4.4 mM was found in scutella on the 7th day of cultivation.

As previously described for roots of pea seedlings (Brunold and Suter, 1989), two enzymes of the sulfate-assimilating pathway, ATP-sulfurylase and APSSTase, were used to identify proplastid fractions from maize root homogenates after sucrose density gradient centrifugation. The proportion of APSSTase activity detected in the proplastid fractions was more than 50% higher than the proportion of ATP-sulfurylase activity (Fig. 4, Table II). The proplastid fractions were clearly separated from mitochondria and microsome fractions, which were localized on the gradients using Cyt *c* oxidase and catalase, respectively, as marker enzymes (fractions 8–14). Part of γEC synthetase activity banded in the proplastid fractions, but most of it was detected on top of the sucrose gradient (Fig. 4A, Table II). Very little of the GSH synthetase activity was measured in the proplastid fractions (Fig. 4B, Table II). A comparably small percentage of GSH synthetase activity can be assigned to mitochondria or microsome fractions (Fig. 4B).

DISCUSSION

Recently, Rauser et al. (1991) reported the occurrence of [^{35}S]GSH but almost no [^{35}S]Cys in scutella, roots, and shoots of maize seedlings 6 h after injecting [^{35}S]Cys into the endosperm. From these findings, they derived a model of GSH synthesis and allocation in maize seedlings: the hydrolysis of sulfur-rich proteins in the endosperm provides free Cys, which is absorbed by the scutella and used for GSH synthesis in this organ. GSH is then transferred through the phloem to the roots and shoots. According to this model, scutella play an important role in GSH synthesis. Our data concerning enzyme distribution in maize seedlings support this model:

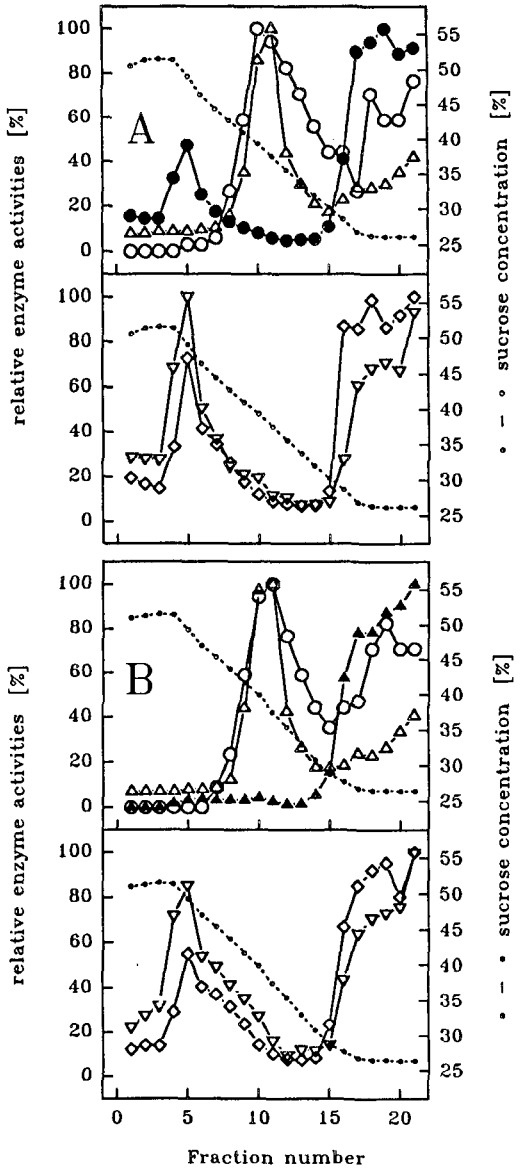

Figure 4. Distribution of γEC synthetase (●, A), GSH synthetase (▲, B), and marker enzyme activity (▽, APSSTase; ◇, ATP-sulfurylase; △, Cyt *c* oxidase; ○, catalase) from root homogenates of 4-d-old maize seedlings after sucrose density gradient separation. For each enzyme, the highest activity detected in a fraction was set as 100%. The values representing 100% of activity (nmol mL^{-1} fraction min^{-1}) were 0.49 for γEC synthetase, 1.1 for GSH synthetase, 0.051 and 0.035 for APSSTase, 1.1 and 0.97 for ATP-sulfurylase, 310 and 300 for Cyt c oxidase, and 27,200 and 27,100 for catalase for the data presented in A and B, respectively.

Table II. *Enzyme activity recovered as percentage of the total activity from maize root homogenates added to sucrose density gradients and as percentage of the recovered activities detected in the fractions containing intact proplastids*

The values belonging to experiments 1 and 2 are derived from the data presented in Fig. 4, A and B, respectively.

Enzyme	Enzyme Activity in Proplastid Fractions		Enzyme Activity Recovered	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
γEC synthetase	17	n.d.[a]	60	n.d.
GSH synthetase	n.d.	3.1	n.d.	73
APSSTase	37	38	41	38
ATP-sulfurylase	24	23	54	44
Cyt c oxidase	2.8	2.0	79	74
Catalase	4.3	3.5	62	63

[a] n.d., Not determined.

scutella from 3- or 7-d-old seedlings contained far more extractable activity of γEC synthetase and GSH synthetase than any other seedling part (Fig. 2). The highest specific activity of both enzymes, calculated on a fresh weight basis, was also measured in scutella (Table I). But roots and shoots also contained extractable γEC and GSH synthetase activity with high levels in 3-d-old seedlings; thus, the GSH provided from the scutella to these seedling parts may be the dominant but not the only source for GSH in the early seedling stage. γEC synthetase is probably inhibited in vivo, however, by the millimolar concentrations of GSH present in the corresponding tissues (Table I), because 2 mM GSH caused a decrease in activity by 70% for the tobacco enzyme in vitro (Hell and Bergmann, 1990). This inhibitory effect is not a general effect of GSH: the maintenance of reducing conditions due to the high GSH concentrations as measured in scutella up to the 11th day of cultivation may also enhance enzymic activities, as suggested for reactions involved in storage protein mobilization (Hay et al., 1991) or fatty acid synthesis (Butt and Ohlrogge, 1991).

The level of GSH present in the entire seedling was roughly constant from the 3rd to the 11th day of cultivation. Thus, as our in vivo data for GSH synthesis in maize seedling roots indicate (Rüegsegger and Brunold, 1992), a comparably low rate of synthesis may be sufficient in vivo to compensate for the GSH lost in degradation processes. In plants exposed to heavy metals, an enhanced demand for GSH is induced, because GSH is used as substrate for the formation of phytochelatins (Grill et al., 1989), the heavy metal-chelating peptides of plants (Rauser, 1990; Steffens, 1990). The scutella may not be able to cover this additional demand: the GSH level declined in the roots of Cd-exposed maize seedlings (Rauser et al., 1991; Meuwly and Rauser, 1992; Rüegsegger and Brunold, 1992), and both the extractable activity of γEC synthetase and GSH synthetase and the in vivo synthesis rate of GSH increased due to Cd (Rüegsegger and Brunold, 1992).

To estimate the intracellular distribution of the enzymes of GSH synthesis in root cells, we assume that in maize APSSTase is localized exclusively in the proplastids, as has been demonstrated for pea roots (Brunold and Suter, 1989). The APSSTase activity on top of the sucrose density gradients would then result from proplastids broken during the extraction and separation procedure. On this assumption, the yield of intact proplastids obtained can be estimated by comparing the APSSTase activity in the proplastid fractions with the total activity of this enzyme on the gradient. According to this estimation, 37 and 38% of the proplastids, respectively, were isolated intact in the experiments presented in Figure 4. These values are comparable to those obtained with pea roots (Brunold and Suter, 1989). When this percentage and the distribution of γEC synthetase activity on the gradient (Fig. 4A, Table II) are used, 46% of this enzyme activity can be calculated to be localized in proplastids of intact root cells.

When the same method of calculation for GSH synthetase is adopted (Fig. 4B, Table II), only 8.2% of its activity may be assigned to this organelle. The prominent accumulation of γEC found in the roots of Cd-treated maize seedlings (Rauser et al., 1991; Meuwly and Rauser, 1992; Rüegsegger and Brunold, 1992) may be caused in part by the lack of sufficient GSH synthetase activity in the proplastids. A comparably low percentage of this enzyme activity may be present in mitochondria or microsomes. The low percentage of GSH synthetase activity found localized in root proplastids is in contrast to that measured in chloroplasts; probably the chloroplasts contain a markedly higher percentage of both γEC synthetase (Lancaster et al., 1989; Hell and Bergmann, 1990) and GSH synthetase (Klapheck et al., 1987; Hell and Bergmann, 1988, 1990) activity to provide the GSH required for the detoxification of oxidizing radicals occurring as a by-product of photosynthetic activity.

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