Glutathione (GSH), the tripeptide γ-Glu-Cys-Gly, plays various important roles in plants. Through its thiol residue, it performs redox reaction, enabling it to be a regulator of redox homeostasis (Foyer and Noctor, 2005). It detoxifies photosynthetically generated hydrogen peroxide as a component of the ascorbate-GSH cycle (Noctor and Foyer, 1998). GSH is polymerized to form phytochelatins, (γ-Glu-Cys)2–11–Gly, that chelate heavy metals and then transport them into the vacuole, where they are metabolically inactive (Cobbett and Goldsborough, 2002). Toxic xenobiotics, including herbicides, are conjugated with GSH by glutathione S-transferases and sequestered into the vacuole (Marrs, 1996). GSH is also involved in controlling cell size and root development by regulating the cell cycle (Vernoux et al., 2000; Xiang et al., 2001). GSH exists in high concentration in plant tissues and acts as a substrate for central biochemicals, particularly Cys, and Cys availability for protein synthesis and as a precursor for metabolites may be related to the rate of GSH degradation (Leustek et al., 2000).

GSH is synthesized from Glu, Cys, and Gly in a two-step, ATP-dependent reaction. First, γ-glutamyl-Cys (γ-EC) is synthesized by γ-EC synthetase (Hell and Bergmann, 1990), and then Gly is incorporated by GSH synthetase (Wang and Oliver, 1996). The activity of γ-EC synthetase is regulated by GSH levels (Hell and Bergmann, 1990; Jez et al., 2004). In Arabidopsis (Arabidopsis thaliana), γ-EC synthetase and GSH synthetase are encoded by single genes, GSH1 (May and Leaver, 1994) and GSH2 (Wang and Oliver, 1996), respectively, and expression of both genes is regulated transcriptionally and translationally by heavy metals, jasmonic acid, and oxidative stress (Xiang and Oliver, 1998). γ-EC synthetase is exclusively localized in the plastids, whereas GSH synthetase, albeit also present in the chloroplasts, is, to a large extent, a cytosolic protein (Wachter et al., 2005). Recently, it was reported that restricting glutathione synthesis to the cytosol is sufficient for normal plant development (Pasternak et al., 2008).

Compared with GSH synthesis, its metabolism is less understood in plants. In animals, the γ-glutamyl cycle is responsible for GSH metabolism (Meister and Goldsmith, 1962). In Arabidopsis, the γ-glutamyl cycle, where GSH is degraded by the sequential reaction of γ-glutamyl transpeptidase (GGT), γ-glutamyl cyclotransferase, and 5-oxoprolinase to yield glutamate (Glu) and dipeptides that are subject to peptidase action. In this study, we examined if GSH is degraded through the same pathway in Arabidopsis (Arabidopsis thaliana) as occurs in mammals. In Arabidopsis, the oxoprolinase knockout mutants (oxp1-1 and oxp1-2) accumulate more 5-oxoproline (5OP) and less Glu than wild-type plants, suggesting substantial metabolite flux through 5OP and that 5OP is a major contributor to Glu steady-state levels. In the ggt1-1/ggt4-1/oxp1-1 triple mutant with no GGT activity in any organs except young siliques, the 5OP concentration in leaves was not different from that in oxp1-1, suggesting that GGTs are not major contributors to 5OP production in Arabidopsis. 5OP formation strongly tracked the level of GSH in Arabidopsis plants, suggesting that GSH is the precursor of 5OP in a GGT-independent reaction. Kinetics analysis suggests that γ-glutamyl cyclotransferase is the major source of GSH degradation and 5OP formation in Arabidopsis. This discovery led us to propose a new pathway for GSH turnover in plants where GSH is converted to 5OP and then to Glu by the combined action of γ-glutamyl cyclotransferase and 5-oxoprolinase in the cytoplasm.
absorbed into the cell and used to reform GSH. γ-Glutamyl transpeptidase (GGT) initiates GSH breakdown and is on the plasma membrane, with its active site in the extracellular space. GGT transfers γ-linked Glu from GSH to water or another amino acid, producing either Glu or a γ-glutamyl amino acid, respectively. The resulting Glu and γ-glutamyl amino acids are transported back into the cell. As originally proposed, the movement of this γ-glutamyl amino acid into the cell was viewed as an important route of amino acid uptake (Meister, 1973), but some authors have presented experimental data suggesting that the most common product of the GGT reaction is free Glu (Hanigan and Pitot, 1985), thus decreasing the role in amino acid transport without affecting its importance in GSH movement. Inside the cell, γ-glutamyl cyclotransferase (GGC) converts any γ-glutamyl amino acids formed into 5-oxoproline (5OP; synonyms: pyrroglutamate and pyrrolidone carboxylate) and the free amino acid. 5-Oxoprolinase (5OPase) hydrolyzes 5OP to Glu in an ATP-dependent reaction. Cys-Gly produced from GSH by GGT is hydrolyzed at the cell surface to Cys and Gly by a dipeptidase before these components reenter the cell. Glu, Cys, and Gly released from GSH are recycled for GSH synthesis in the cell by γ-EC synthetase and GSH synthetase. Activities of GGT, GGC, and 5OPase are highest in the kidney along with other secretory tissues. In the kidney, GSH is filtered from the blood into the excretory stream. The γ-glutamyl cycle is responsible for reabsorbing the GSH by first triggering its breakdown and then, after reabsorption of the component amino acids, catalyzing their conversion back to GSH. This idea is supported in GGT knockout mice that showed glutathionuria, Cys deficiency, and growth and reproductive defects that can be reversed by feeding acetylcysteine (Lieberman et al., 1996; Harding et al., 1997; Kumar et al., 2000).

In plants that lack a formal excretory system, the presence of a γ-glutamyl cycle has yet to be determined. All of the enzymes in this cycle are reported in plants except the Cys-Gly dipeptidase. Soluble and bound GGT activity has been detected in tomato (Solanum lycopersicum), onion (Allium cepa), radish (Raphanus sativus), and Arabidopsis (Martin and Slovin, 2000; Storozhenko et al., 2002; Nakano et al., 2004; Shaw et al., 2005; Grzam et al., 2007; Martin et al., 2007; Ohkama-Ohtsu et al., 2007a, 2007b). Recently, we analyzed the functions of Arabidopsis GGT proteins using knockout mutants (Ohkama-Ohtsu et al., 2007a, 2007b). There are three functional GGT proteins in Arabidopsis, GGT1, GGT2, and GGT4. GGT4 (At4g29210) was originally called GGT 3 in our earlier papers (Ohkama-Ohtsu et al., 2007a, 2007b) but was renamed here to agree with the nomenclature of Martin et al. (2007), who simultaneously discovered it function (Grzam et al., 2007). GGT1 mitigates oxidative stress by degrading oxidized GSH in the extracellular space, and GGT2 (also found in the apoplast) may be involved in GSH transport into siliques (Ohkama-Ohtsu et al., 2007a). GGT4 is located in the vacuole, where it is responsible for the degradation of GSH conjugates formed by glutathione S-transferase reactions (Ohkama-Ohtsu et al., 2007b).

Less is known about the function of 5OPase and GGC in plants. 5OPase activity was first detected in tomato by Mazelis and Pratt (1976). They showed conversion of isotope-labeled 5OP to Glu in...
both monocots and dicots. Plant 5OPase was first purified from wheat germ (*Triticum aestivum*) by Mazelis and Creveling (1978), who also observed 5OPase activity in various organs, including roots and seeds, from a range of species. Subcellular localization of 5OPase was analyzed in cultured tobacco (*Nicotiana tabacum*) cells by Rennenberg et al. (1981). Almost all of the activity was reported to be in the soluble cytoplasmic fraction. Feeding isotope-labeled GSH to cultured tobacco cells demonstrated that 5OP is a degradation product of GSH (Rennenberg et al., 1980). GGC was studied in tobacco suspension cultures by Steinkamp and Rennenberg (1985, 1987), who suggested that this soluble enzyme is also localized in the cytoplasm.

Here, we studied 5OP synthesis and degradation using knockout mutants of the Arabidopsis 5OPase. Surprisingly, millimolar concentrations of 5OP accumulate in these knockout mutants, suggesting substantial carbon and nitrogen flow through 5OP to Glu in Arabidopsis. Combining the 5OPase with GGT knockouts demonstrated that while 5OP is made from GSH and γ-EC, it is not derived via the action of GGT. Furthermore, GGC enzyme kinetic analyses suggested that GSH is a more important source of 5OP than γ-EC under physiological conditions. These results raise questions about the importance of the traditional γ-glutamyl cycle in plants and identify a new pathway that may also be important in other organisms.

## RESULTS

### The Arabidopsis 5OPase Gene

In Arabidopsis, a single gene (*At5g37830*) shows high homology to 5OPase from animals and was named *OXOPROLINASE1* (*OXP1*). The translated sequence of Arabidopsis *OXP1* is 57% identical and 71% to 72% similar to the protein from the sequences of *Rattus norvegicus*, *Mus musculus*, *Homo sapiens*, and *Bos taurus*. High similarity to animal 5OPases is observed throughout the Arabidopsis protein, including the N terminus (Supplemental Fig. S1), suggesting that Arabidopsis 5OPase, like the animal proteins (Meister and Larsson, 1995), localizes to the cytoplasm. This was supported by computer prediction using PSORT (http://psort.hgc.jp/) and TargetP (http://www.cbs.dtu.dk/services/TargetP/) and agrees with the localization in tobacco (Rennenberg et al., 1981).

Expression of the *OXP1* gene was analyzed using real-time PCR. *OXP1* transcript was observed in all organs examined, with the highest levels in flowers and 3-week-old rosette leaves and the lowest in stems (Fig. 2).

### The *oxp1* Mutants

Knockout mutants are effective tools for analyzing the function of genes in planta. Two allelic mutants disrupting *OXP1* were obtained and designated *oxp1-1* and *oxp1-2* (Fig. 3A). *oxp1-1* is in the Columbia background with a T-DNA insertion in the second exon of *OXP1*. *oxp1-2* is in the Wassilewskija background and also has a T-DNA insertion in the second exon. *OXP1* mRNA was not detected by reverse transcription (RT)-PCR analysis either in the *oxp1-1* or *oxp1-2* plants (Fig. 3B). In order to verify that *oxp1* is the only gene encoding 5OPase activity, we measured the ability of dialyzed protein extracts from wild-type and *oxp1-1* plants to convert 5OP into Glu (Table I). Both wild-type and *oxp1-1* extracts formed a small amount of Glu (possibly released from proteins) in the absence of added 5OP, but only the wild-type line was able to convert added 5OP to Glu. The lack of detectable 5OPase activity in the *oxp1-1* mutant plants verifies that *OXP1* is the only gene encoding 5OPase in Arabidopsis and that no other enzyme catalyzes this reaction.

Both the *oxp1-1* and *oxp1-2* plants were grown on soil until they set seeds. The *oxp1-2* mutant flowered approximately 5 d earlier than Wassilewskija wild-type plants, but the early-flowering phenotype is unlikely to be due to disruption of the *OXP1* gene because this phenotype was not observed in *oxp1-1*. Root growth was not significantly different from that of wild-type plants in both *oxp1-1* and *oxp1-2* when they were grown vertically on agar plates for 14 d (data not shown). No morphological phenotypes were observed in *oxp1-1*, so this allele was used in most metabolism studies. Neither mutant line showed differences in thiol levels and composition from wild-type plants, which is not unexpected given that this disruption occurred relatively late in the GSH catabolic pathway.
5OP Accumulates in the oxp1 Mutants

5OP was measured by HPLC (Nishimura et al. 2001) after ethanolic plant extracts were passed through a Dowex 50 (H+) column (Orlowski et al., 1969). 5OP distributions in both Columbia wild-type and oxp1-1 plants are shown in Figure 4A. In leaves, 5OP was not detected in wild-type plants, while it accumulated to approximately 2 μmol g⁻¹ fresh weight in the oxp1-1 plants, where its further metabolism was blocked. In stems, 5OP was undetectable in wild-type plants but accumulated to 4 μmol g⁻¹ fresh weight in the mutant. In sink tissues, roots, flowers, and siliques, 5OP was barely detectable in wild-type plants but accumulated to nearly 10 μmol g⁻¹ fresh weight in the oxp1-1 plants. Very similar results were seen in leaves and flowers of the oxp1-2 plants (Fig. 4B). Given the lack of apparent phenotype, the accumulation of high 5OP levels in oxp1-1 suggests that this intermediate is not toxic. The identity and amount of 5OP measured by HPLC was confirmed using capillary electrophoresis time-of-flight mass spectrometry (CE-TOF/MS) method confirmed a background level of 0.5 ± 0.1 μmol g⁻¹ fresh weight (n = 6).

Glu Concentrations Decrease in the oxp1 Mutants

5OPase hydrolyzes 5OP to Glu; thus, analysis of Glu levels in the oxp1 plants would provide some insights into the contribution of this reaction to Glu formation. Glu concentrations in 20- and 30-d-old rosette leaves, 50-d-old cauline leaves, and flowers were significantly (14%–30%) lower in the oxp1-1 plants compared with wild-type plants (Fig. 4C). In all other organs except roots, Glu concentrations were also lower in oxp1-1 plants, but the differences were not statistically significant. The same results were observed in leaves and flowers from oxp1-2 plants (Fig. 4D). These results suggest that 5OPase contributes up to 30% of the steady-state Glu level in Arabidopsis.

Growth studies showed that despite the decreased Glu levels, the oxp1 plants were no more sensitive to nitrogen deficiency than wild-type plants and that the levels of the key amino acids Asp, Ser, Gln, Gly, Thr, Arg, and Ala were unchanged (data not shown).

5OP Concentrations Are Not Changed by ggt Mutations

In order to determine the relative contributions of extracellular GGT1 and vacuolar GGT4 to 5OP production, the ggt1-1/oxp1-1 (double), ggt4-1/oxp1-1 (double), and ggt1-1/ggt4-1/oxp1-1 (triple) mutants were constructed by crossing mutant plants. Since GGT2 is only expressed in young siliques (Ohkama-Ohtsu et al., 2007a), it was not included in this study. As shown in Supplemental Table S1 and our previous reports (Ohkama-Ohtsu et al., 2007a, 2007b), GGT activity in the leaves from ggt1 and ggt4 plants was about 10% and 90% of wild-type GGT activity, respectively, while the ggt1/ggt4 double mutant had no detectable GGT activity. As the ggt1-1 and ggt4-1 plants are in the Landsberg background (Ohkama-Ohtsu et al., 2007a, 2007b) and oxp1-1 is in Columbia, oxp1-1 was crossed to Landsberg wild-type plants, and a mixture of F3 seeds derived from five F2 plants carrying the homozygous oxp1-1 mutation was used as a control. 5OP concentrations in leaves of the 20-d-old ggt1-1/oxp1-1, ggt4-1/oxp1-1, and ggt1-1/ggt4-1/oxp1-1 plants (Table II) were not significantly different from those in the oxp1-1 plants. These results clearly demonstrate that GGTs are not in the major pathway for 5OP production in Arabidopsis.

5OP Decreases in Plants with Less GSH or γ-EC

Having demonstrated that GGTs were not significant contributors to 5OP formation, this means that the 5OP was not being produced from the γ-glutamyl amino acid that can be produced by the GGT reaction in the γ-glutamyl cycle (Meister and Larsson, 1995). The roles of the major endogenous γ-glutamyl amino acids, GSH and γ-EC, in 5OP production were then determined. To know whether 5OP came from GSH or

| Line                  | With Substrate | Without Substrate |
|-----------------------|----------------|-------------------|
|                       | nmol mg⁻¹ protein min⁻¹ | 0.04 ± 0.02 |
| Columbia wild type    | 0.39 ± 0.04     | 0.04 ± 0.02       |
| oxp1-1                | 0.04 ± 0.02     | 0.03 ± 0.01       |

*Significant difference between with and without substrate (one-tailed Student’s t test, P < 0.05).
γ-EC in Arabidopsis, an inhibitor of γ-EC synthetase, buthionine sulfoximine (BSO), was supplied to \( \text{oxp1-1} \) mutant plants grown in liquid culture. GSH and 5OP concentrations were determined in 14-d-old seedlings that were treated with BSO for 1 to 4 d. Control plants were cultured without BSO for 14 d. In a preliminary experiment, GSH concentrations were higher in plants treated with BSO for 3 or 4 d compared with plants treated for 1 or 2 d, so fresh BSO was added every 2 d. Compared with nontreated plants, GSH and γ-EC concentrations were substantially decreased in plants treated with BSO. The 5OP level was lowered in all plants treated with BSO, and the decrease was significant after 2 d (Table III). The decrease in the percentage of 5OP is smaller than that for GSH or γ-EC (6% for 5OP versus 81% for GSH and 54% for γ-EC at day 1) because of the high background 5OP concentration in \( \text{oxp1-1} \) plants at the time of BSO addition and the fact that no further 5OP metabolism occurs in this mutant. Although the ratio is different, the absolute decreases in 5OP and GSH levels at day 1 are very similar.

This correlation between GSH/γ-EC and 5OP levels was further confirmed in the \( \text{cad2-1/oxp1-1} \) double mutants. \( \text{cad2-1} \) (Columbia background) has a defect in the γ-EC synthetase gene, and both GSH and γ-EC levels were less than half compared with wild-type plants (Cobbett et al., 1998). In agreement with the previous report, GSH and γ-EC concentrations in leaves of the \( \text{cad2-1/oxp1-1} \) double mutant were 21% and 39% of those in the \( \text{oxp1-1} \) single mutant (Table IV). 5OP concentration in the \( \text{cad2-1/oxp1-1} \) double mutants was decreased to 46% of that in the \( \text{oxp1-1} \) single mutant, again demonstrating a tight coupling between GSH/γ-EC and 5OP levels.

Both the results with BSO and with the \( \text{cad2-1/oxp1-1} \) plants suggested that 5OP is produced from GSH and/or γ-EC, although the relative contribution of each could not be determined. To address this issue, an experiment was designed where GSH levels could be increased without increasing γ-EC. To do this, liquid-cultured \( \text{oxp1-1} \) plants were supplemented with 2 mM GSH and incubated for 20 h. Compared with plants without GSH supplementation, the 5OP concentration was significantly increased in the GSH-fed \( \text{oxp1-1} \) mutants (Table V). In the GSH-treated \( \text{oxp1-1} \) plants, GSH, Cys-Gly, and Cys were also increased, but γ-EC was not changed. GSH was also provided to the \( \text{ggt1-1/ggt4-1/oxp1-1} \) triple mutant to eliminate the possibility that GSH was converted to γ-glutamyl amino acids by GGTs that then acted as 5OP precursors. In the \( \text{ggt1-1/}

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**Figure 4.** 5OP and Glu concentrations in wild-type (WT) and \( \text{oxp1} \) plants. A, 5OP in Columbia (Col) wild-type and \( \text{oxp1-1} \) plants. B, 5OP in Wassilewskija (Ws) wild-type and \( \text{oxp1-2} \) plants. C, Glu in Col wild-type and \( \text{oxp1-1} \) plants. D, Glu in Ws wild-type and \( \text{oxp1-2} \) plants. Roots of liquid-cultured 12-d-old plants, rosette leaves from soil-grown 20-, 30-, and 50-d-old plants, and cauline leaves, stems, and flowers from soil-grown 50-d-old plants and siliques (2–4 d after flowering [DAF]) were harvested from Col wild-type and \( \text{oxp1-1} \) plants and assayed for 5OP and Glu. The means ± SD of three (5OP) or four (Glu) biological replicates are shown. Asterisks indicate significant differences between Col wild-type and \( \text{oxp1-1} \) plants or between Ws wild-type and \( \text{oxp1-2} \) plants. (\( P < 0.05 \), one-tailed Student’s \( t \) test). N.D., Not detected.
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Table II. The effect of the ggt1 and ggt4 mutations on 5OP concentrations in oxp1-1 Arabidopsis leaves

| Line                        | 5OP            |
|-----------------------------|----------------|
| oxp1-1 (Landsberg)          | 1,370 ± 30     |
| ggt1-1/oxp1-1               | 1,440 ± 110    |
| ggt4-1/oxp1-1               | 1,390 ± 60     |
| ggt1-1/ggt4-1/oxp1-1        | 1,340 ± 50     |

The means ± SD of five biological replicates are shown. None of these values was significantly different by a one-tailed Student’s t test (P > 0.05).

ggt4-1/oxp1-1 triple mutant, 5OP was also increased following GSH treatment, while the γ-EC levels were not (Table V). The increases in GSH and 5OP were similar in magnitude.

Finally, the ability of plants to degrade GSH in the absence of GGTs was shown by adding BSO to the ggt1-1/ggt4-1 double knockout and wild-type plants. GSH concentrations in liquid-cultured plants were determined at 8, 12, 16, and 20 h after addition of BSO. As shown in Table VI, the rate of GSH degradation was similar between wild-type and ggt1-1/ggt4-1 plants, suggesting that under these conditions the GGTs were not contributing significantly to GSH turnover.

Taken together, these results demonstrate that GSH is the major source for 5OP synthesis, this reaction does not involve GGT, and GGT is not the prominent reaction of GSH turnover.

GGC Enzyme Kinetics with GSH and γ-EC

The enzyme kinetics of GGC were studied to determine if this gene corresponds to GGC activity. GGC activity (At4g25720; Supplemental Fig. S2, A and B) to examine if GGC is not encoded by this gene. Furthermore, we examined if QCT contributes to 5OP production. There was no significant difference in 5OP concentration between oxp1-2 and the At4g25720/oxp1-2 mutant (Supplemental Fig. S2C), suggesting that GGC is not encoded by this gene. Furthermore, we examined if QCT contributes to 5OP production. There was no significant difference in 5OP concentration between oxp1-2 and the At4g25720/oxp1-2 mutant (Supplemental Fig. S2D), indicating that At4g25720 is not involved in the major pathway for 5OP production.

DISCUSSION

In this study, we analyzed the synthesis and metabolism of 5OP in Arabidopsis using knockout mutants for 5OPase, the enzyme responsible for 5OP conversion to Glu. Disruption of OXP1, the gene for 5OPase, resulted in a large increase in 5OP levels and in some organs significant decreases in Glu levels. It was interesting how much impact blocking 5OP metabo-

Table III. 5OP and thiol concentrations in BSO-treated oxp1-1 seedlings

| Treatment | 5OP | GSH | γ-EC | Cys |
|-----------|-----|-----|------|-----|
| No BSO    | 5,280 ± 450 | 369 ± 55 | 12.2 ± 2.4 | 37 ± 5 |
| 1 d       | 4,990 ± 570 | 71 ± 26a | 5.6 ± 1.9a | 57 ± 4a |
| 2 d       | 4,740 ± 240a | 36 ± 5a | N.D. | 52 ± 10a |
| 3 d       | 3,970 ± 390a | 27 ± 3a | N.D. | 62 ± 9a |
| 4 d       | 3,160 ± 280a | 31 ± 4a | N.D. | 64 ± 5a |

aSignificant difference from nontreated plants (one-tailed Student’s t test, P < 0.05).
Table IV. The effect of the cad2 mutation on 5OP and thiol concentrations

| Line               | 5OP          | GSH          | γ-EC         | Cys          |
|--------------------|--------------|--------------|--------------|--------------|
| 2m M 6,060         | N.D.         | N.D.         | N.D.         | N.D.         |
| 4,150              | 354 ± 29     | 14.8 ± 1.8   | N.D.         | 47 ± 8       |
| 2m M 6,150         | 670 ± 40°    | 61 ± 6°      | 4.8 ± 1.72°  | 60 ± 15°     |

*aSignificant difference between oxp1-1 and cad2-1/oxp1-1 plants (one-tailed Student’s t test, P < 0.05).

Table V. The effect of GSH application on 5OP concentrations

| Line               | GSH Added | 5OP          | GSH          | γ-EC         | Cys-Gly       | Cys          |
|--------------------|-----------|--------------|--------------|--------------|---------------|--------------|
| 2m M 6,060         | 0 m M     | 4,880 ± 990  | 354 ± 29     | 14.8 ± 1.8   | N.D.          | 47 ± 8       |
| 2 m M 6,150        | 6,150 ± 590°| 1,519 ± 144°| 14.8 ± 1.9   | 10.4 ± 0.9   | 293 ± 54°    | N.D.         |
| 2 m M 6,060        | 4,150 ± 340| 368 ± 49     | 16.0 ± 2.1   | N.D.         | 43 ± 8        |
| 2 m M 6,060        | 6,060 ± 570°| 1,347 ± 55°  | 15.1 ± 0.8   | N.D.         | 90 ± 10°      |

*aSignificant difference from plants without GSH (one-tailed Student’s t test, P < 0.05).

lism had on steady-state Glu levels. While the lack of effect of the oxp1-1 mutant on Glu levels in roots suggests that this tissue can compensate for the disruption in 5OP metabolism, the 14% to 30% decrease in Glu levels in leaves, flowers, and siliques suggests that 5OP is a major source of Glu formation in these organs. Future flux studies will be necessary to estimate the actual amount of Glu derived from 5OP.

In the oxp1 mutants, 5OP accumulated to millimolar levels in all tissues tested, with the highest levels in roots, flowers, and young siliques. Genetic and metabolic experiments demonstrated that GSH is the predominant source of 5OP. It is possible that γ-EC is also contributing, but since this intermediate normally occurs at concentrations that are 10- to 20-fold lower than GSH and since 5OP synthesis seemed to track GSH level and not γ-EC concentration, its role is likely to be relatively minor. Assuming exponential growth and a constant rate of 5OP synthesis, oxp1 leaves accumulated 5OP at a rate of about 40 nmol g⁻¹ fresh weight h⁻¹ (Fig. 4), a value that agrees reasonably well with observed in vivo rates of GSH breakdown when GSH synthesis is blocked by BSO, about 30 nmol g⁻¹ fresh weight h⁻¹ (Table III). The similarity between rates of GSH degradation and 5OP accumulation suggests that the majority of GSH is metabolized through 5OP and that the major portion of 5OP is derived from GSH. These results also suggest that mechanisms for GSH degradation that do not proceed through 5OP, such as the GSH hydrolase activity of GGT or the newly discovered DUG hydrolase system in yeast (Ganguli et al., 2007), are of limited importance in total GSH turnover in plants.

GGC kinetic analyses supported the idea that GSH is a more important source of 5OP than γ-EC (Fig. 5). Although the Vₘₐₓ for GGC was 3.3 times higher with γ-EC than with GSH, the Kₘ was 2.9 times lower with GSH than with γ-EC, suggesting that GGC has a higher affinity for GSH than γ-EC. GGC reaction rates were calculated using the best available estimates of cytoplasmic GSH or γ-EC concentrations. Fricker et al. (2000) determined cytoplasmic GSH concentration in the roots of intact Arabidopsis seedlings after labeling with monochlorobimane to give fluorescent GSH S-bimane conjugates and imaging using confocal laser scanning microscopy. Thecytoplasmic GSH concentration they measured was 2 to 3 mM. Assuming that γ-EC concentration in the cytoplasm is also proportionately higher than that in whole tissues, the cytoplasmic γ-EC concentration would be about 0.1 mM. Using these values with the kinetic constants we determined in the Michaelis-Menten equation, the GGC reaction rates in the cytoplasm were calculated as 0.11 nmol mg⁻¹ protein min⁻¹ for GSH and 0.02 nmol mg⁻¹ protein min⁻¹ for γ-EC, suggesting that 5.5 times more 5OP is synthesized from GSH than from γ-EC in vivo. The GGC reaction rate with GSH in the cytoplasm is equivalent to 60 nmol g⁻¹ fresh weight h⁻¹, a value that compares extremely well with the rates of GSH breakdown when GSH synthesis is blocked by BSO and of 5OP formation in the oxp1 mutants. These findings all support the suggestion that GGC is responsible for most GSH degradation in vivo.

The protein and gene corresponding to GGC are unidentified in plants, although QCT has been eliminated as a possibility (Supplemental Fig. S2). Specific identification of the Arabidopsis gene encoding GGC and analysis of the mutant are necessary to verify its function and to test our hypothesis that it is the major source of GSH metabolism in vivo. Recently, the gene...
encoding GGC was identified from humans (Oakley et al., 2008). Homology searches using the human GGC sequence did not identify any similar proteins in plants (Oakley et al., 2008), although GGC activity has been detected in tobacco (Steinkamp and Rennenberg, 1985, 1987) and Arabidopsis (this study). This suggests that the plant and animal enzymes are too highly diverged to be recognized by this method. Human GGC contains a BtrG-like fold that might be a signature feature of GGCs. A small family of Arabidopsis proteins with unknown function also contains this fold (de la Cruz et al., 2008) and may include the Arabidopsis GGC.

The lack of involvement of GGT as a major component of GSH metabolism agrees with the observations in our GGT knockout plants, where no significant changes in GSH levels were measured in any tissues except in the apoplast (Ohkama-Ohtsu et al., 2007a). While some GSH metabolism occurs through the GGT enzymes in plants and this metabolism is physiologically important in plants, it is clearly a minor portion of total GSH degradation.

Therefore, there is a fundamental difference in the ways plants and animals metabolize GSH. In animals, GSH is predominantly metabolized in the nephron tubes of the kidneys. GGT catalyzes this step, most likely releasing Glu and Cys-Gly, with the latter being hydrolyzed outside of the cell. The hydrolysis of GSH into component amino acids initiates its rapid transport into the cell, preventing the loss of these amino acids in the excretory system. In plants, the GGT reaction is a minor component under our experimental conditions. Rather, GSH is rapidly degraded through 5OP by GGC. The differences between the plants and animals may reflect the lack of an excretory system in plants. Having demonstrated this variation of the γ-glutamyl cycle in plants, where 5OP is formed from GSH by GGC and not GGT, it is interesting to ask if GGC fills the same role in animals. We were only able to prove this pathway in plants because of the possibility of creating organisms that lacked both GGT and 5OPase activity. This has not been undertaken in animals.

Although both GGT and GGC hydrolyze γ-glutamyl bonds, the lack of GGT activity in the ggt1/ggt4 double mutant indicates that GGT activity was distinct from GGC activity in our GGT activity assay (Ohkama-Ohtsu et al., 2007b). This was supported by the fact that human GGC did not metabolize γ-glutamyl-p-nitroanilide, the substrate for the GGT assay (Orlowski et al., 1969), and that the product of the reaction is 5OP and not Glu.

Figure 1B shows our current model for GSH degradation in plants, in which cytoplasmic GSH is converted to 5OP in a GGT-independent manner. While we have generally presented the conversion of GSH to 5OP by GGC as a one-step process, it is also possible to postulate an intermediate. The higher Vmax with γ-EC might support the notion that γ-EC or some other γ-glutamyl amino acids could be preferred GGC substrates and that a two-step conversion occurs. High enough levels of γ-EC or other γ-glutamyl amino acids to drive this reaction have not yet been observed in Arabidopsis. Phytochelatins could also act as 5OP

### Table VI. The rate of GSH degradation in Landsberg wild-type and ggt1-1/ggt4-1 mutant plants

| Line               | BSO  | GSH     | γ-EC      | Cys     |
|--------------------|------|---------|-----------|---------|
|                    | 0 mM | 443 ± 43| 10.0 ± 1.0| 40 ± 8  |
| Landsberg wild type| 1 mM, 8 h | 297 ± 24 | 8.6 ± 1.9  | 42 ± 10 |
|                    | 1 mM, 12 h | 197 ± 52 | 7.5 ± 2.0  | 69 ± 5  |
|                    | 1 mM, 16 h | 132 ± 19 | 7.4 ± 2.5  | 80 ± 10 |
|                    | 1 mM, 20 h | 113 ± 27 | 6.3 ± 0.9  | 105 ± 24|
| ggt1-1/ggt4-1      | 0 mM | 404 ± 49| 13.4 ± 2.9 | 39 ± 9  |
|                    | 1 mM, 8 h | 267 ± 73 | 9.2 ± 0.4  | 48 ± 10 |
|                    | 1 mM, 12 h | 217 ± 58 | 8.6 ± 1.9  | 71 ± 2  |
|                    | 1 mM, 16 h | 149 ± 23 | 8.6 ± 0.9  | 86 ± 14 |
|                    | 1 mM, 20 h | 105 ± 20 | 8.5 ± 1.1  | 116 ± 26|

Ten-day-old liquid-cultured plants were supplemented with BSO at 1 mM and incubated for 8, 12, 16, or 20 h, then concentrations of thiols were determined. The means ± SD of five biological replicates are shown.
precurors. This would require that GSH is first converted to phytochelatins by phytochelatin synthase before GGC removes the N-terminal γ-glutamyl group to make SOP. Recently, the cytosolic hydrolysis of GSH conjugates to γ-EC conjugates by phytochelatin synthase was reported (Beck et al., 2003; Blum et al., 2007). Since phytochelatin synthase requires a heavy metal, this does not seem likely to be important in our in vitro GGC assay.

While GSH degradation may have a number of physiological functions, one purpose could be to provide Cys for protein synthesis and as a precursor for numerous metabolites. GSH is the major storage and transport form for Cys, and it must be broken down in order to release the amino acid (Leustek et al., 2000). In the future, it will be interesting to know how the futile cycles between GSH synthesis and degradation, which appear to be in the same tissues, are controlled.

**MATERIALS AND METHODS**

**Plant Materials**

Unless otherwise indicated, Arabidopsis (Arabidopsis thaliana) plants were grown at 22°C with a 24-h photoperiod (150 μmol m⁻² s⁻¹). Liquid culture was performed as described (Xiang and Oliver, 1998). Root length measurement on agar plates and nitrogen-deficient treatment in hydroponics culture were as described by Okhama-Ohtsu et al. (2007b).

The exp1-1 mutant (ecotype Columbia) was provided by the Salk Institute (Alonso et al., 2003) and obtained from the Arabidopsis Biological Resource Center (stock no. SALK_078745). The exp1-1 mutant is a result of a T-DNA insertion into the second exon of the OXP1 gene. The homozygous plants containing the insert were screened by PCR using the gene-specific primers OXP1-1F (5'-CAAGCTTCTGACCACTCACTATCA-3') and OXP1-1R (5'-GGGAACTACTCTTGGCAACGAAT-3') and the T-DNA left-border primer pROK6 (5'-GCTTGGCTCTTCTCCCTTCTTCT-3'; Lin and Oliver, 2008).

The exp1-2 mutant (ecotype Wassilewskija) was provided by INRA (http://dbsgap.versailles.inra.fr/publiclines/; stock no. EYU82). The exp1-2 mutant is a result of a T-DNA insertion in the second exon of the OXP1 gene. The homozygous plants containing the insert were screened by PCR using the gene-specific primers OXP1-2F (5'-CCGAGGCTGCTCCAGCAGG-3') and OXP1-2R (5'-GATGCGAGTCATGATTCT-3') and the T-DNA left-border primer LB4 (5'-GGTGGTGCAGTTGGCAACAGATG-3'; http://www.ggb.versailles.inra.fr/en/snpq/equipes/liviers/FST_information.html).

The Atgk25720 mutant (ecotype Wassilewskija) was provided by INRA (stock no. EYH83). The Atgk25720 mutant is a result of a T-DNA insertion in the first exon of Atgk25720. The homozygous plants containing the insert were screened by PCR using the gene-specific primers 5'-CGATGGACTCCATGCAGG-3' and 5'-GGCCGACGAGGAAGTGC-3' and the T-DNA left-border primer LB4 described above.

Thiols were extracted, reduced with dithiothreitol (DTT), and quantified by HPLC as their monobromobimane derivatives (Okhama-Ohtsu et al., 2007a).

**Metabolite Analyses**

**HPLC Analyses**

As 5OP is known to be chemically generated by heat or weak acid from Glu or Gln, plant tissues were homogenized in ice-cold 80% ethanol. After centrifugation at 16,000g for 10 min, the supernatant was collected and the ethanol was removed by vacuum evaporation at room temperature. The residue was dissolved in 2 mL g⁻¹ tissue of 50 mM Tris-HCl, pH 8.0. After centrifugation at 16,000g for 10 min, 60 μL of the supernatant was passed through a 0.8-μL Dowex 50-X16 column (Orolevich et al., 1969). The column was washed with deionized water until 1.6 mL of effluent was collected. The effluent was dried under vacuum, and the residue dissolved in 60 μL of deionized water.

5OP was determined using HPLC as described (Nishimura et al., 2001) with modifications. The HPLC system used a C18 column (4.6 mm × 150 mm; Alltech) and a mobile phase of 2% (v/v) aqueous perchloric acid at 1.0 mL min⁻¹. SOP was detected at 210 nm at 5.2 min (Supplemental Fig. S3). 5OP levels were undetectable in leaf extracts from wild-type plants but detected in leaf extracts from the exp1-1 mutant plants (Supplemental Fig. S3). Internal standards were used to confirm the SOP peak and the efficiency of the extraction method (Supplemental Fig. S3). No Glu or Gln was converted to SOP in this method.

This mixture was extracted, reduced with dithiothreitol (DTT), and quantified by HPLC as their monobromobimane derivatives (Okhama-Ohtsu et al., 2007a).

Amino acid analysis was done following extraction in cold 80% ethanol. After centrifugation at 16,000g for 10 min, the supernatant was removed and dried under vacuum, dissolved in deionized water, and analyzed by reverse-phase HPLC using the AccQ Fluor Reagent Kit (Waters). Glu did not separate from Asn and was measured using a l-Glu assay kit (Seikagaku) and confirmed by CE-TOF/MS.

**CE-MS Analyses**

Frozen Arabidopsis seedlings were homogenized with Zirconia beads using a Mixer Mill (Retsch) at 27 Hz for 3 min. Twenty volumes of methanol (20 μL g⁻¹ fresh weight) including 8 μL internal standard, Met sulfone, that was used for compensation of the peak area after CE-MS analysis, was added, and again the mixture was homogenized at 27 Hz for 1 min. The sample solution was then centrifuged at 20,400g for 3 min at 4°C. Five hundred microliters of chloroform and 200 μL of water were added to the supernatants. This mixture was vortexed for 3 min and centrifuged at 20,400g for 3 min at 4°C. The upper layer was evaporated for 30 min at 45°C by a centrifugal concentrator and then separated into two layers. The upper layer (100–200 μL) was centrifugally filtered through a Millipore 5-kD cutoff filter at 9,100g for 90 min. The filtrate was dried for 120 min by a centrifugal concentrator. The residue was dissolved into 20 μL of water containing a reference compound (3-aminoarylidine). The enzyme reaction mixtures were also prepared as described above. The final solution (20 μL) was used to quantify the contents of 5OP, Glu, and Gln by cation analysis using CE-MS. The CE-MS system and conditions were as described by Watanabe et al. (2008). Quantifications were performed using calibration curves of each compound.

The in vivo rates of 5OP synthesis in exp1-1 and GSH degradation in the presence of BSO were calculated as δC/δt = r − μc, where r = rate of synthesis or degradation (nmol g⁻¹ fresh weight h⁻¹), c = amount of chemical (nmol), δt = change in time, and μ = specific growth rate (h⁻¹).

**RT-PCR and Quantitative Real-Time PCR**

Total RNA was treated with DNase (Invitrogen; http://www.invitrogen.com) and reverse transcribed using SuperScript II reverse transcriptase (Invitrogen) and oligo(dT)₁₂ according to the manufacturer’s instructions.

Real-time PCR analysis was carried out using the iCycler iQ real-time PCR detection system (Bio-Rad; www.bio-rad.com) and iQ SYBR Green Supermix (Bio-Rad) as recommended by the manufacturer. Sequences of primers used in RT-PCR were 5'-GAACCCATCTTGCAGGCG-3' and 5'-CAGAACCCGATCCATCTTC-3' for OXP1 and 5'-GGCCGACGAGGAAGTGC-3' and 5'-CCATCGTGACGGAACAGGTTGG-3' for OXP3. Primers were designed using Primer3 (Rozen and Skaletsky, 2000). The ActIN8 gene (An et al., 1996) was chosen as a control, and its expression was determined using primers as described by Goto and Naito (2002).

**5OPase Assay**

The 5OPase activity assay was performed as described by Mazelis and Creveling (1978) with modifications. Leaves from 20-d-old plants were homogenized in ice-cold (2 mL g⁻¹ fresh weight) 20 mM HEPES (pH 7.4) containing 5 mM DTT and then centrifuged for 10 min at 16,000g, 4°C. The supernatant solution was dialyzed against 20 mM HEPES (pH 7.4) overnight at 4°C.
4°C before the S0Pase assay. S0Pase activity was determined at 30°C in an assay mixture of 100 μM Na glycinate, pH 9.5, 5 μM ATP, 2.5 μM MnCl₂, 2.5 μM MgCl₂, 20 μM (NH₄)₂SO₄, 5 μM DTT, and 2.5 μM S0P in a total volume of 200 μL. Under these conditions, S0Pase activity was constant for at least 60 min. As a negative control without substrate, deionized water replaced S0P. The reaction was stopped by adding 20 μL of 1 M aceitic acid and heating for 5 min at 100°C. The heated reaction mixture was centrifuged, and an aliquot of the supernatant solution was assayed for GL by CE-TOF/MS. The conditions of CE-TOF/MS were the same as those used for S0P determination described above.

**GGC Activity Assay**

The GGC activity assay was performed as described by Steinack and Remenof (1985, 1987) with modifications. Ten-day-old liquid-cultured ggt1-1/ggt14-1 seedlings were homogenized to a fine powder in liquid nitrogen, then suspended in 5 mL g⁻¹ tissue of a solution consisting of 50 μM Tris-HCl, pH 8.0, and 5 μM Z-mercaptoethanol. After centrifugation at 13,000g and 4°C for 15 min, the supernatant was brought to 85% saturation with solid ammonium sulfate. The precipitate was collected by centrifugation at 13,000g and 4°C for 15 min and dissolved in a minimum volume of 50 μM Tris-HCl, pH 8.0. The enzyme solution was dialyzed against 50 μM Tris-Cl, pH 8.0, at 4°C for 2.5 mM MgCl₂, 20 mM (NH₄)₂SO₄, 5 mM DTT, and 2.5 mM 5OP in a total volume for 5 min at 100°C. Under these conditions, GGC activity was constant for 60 min with up to 0.38 mg of GGT1-1 protein. The heated reaction mixture was centrifuged, and an aliquot of the supernatant solution was assayed for GGT activity by CE-TOF/MS. The reaction was stopped by adding 20 μL of 200 mM acetic acid and heating for 5 min. The heat reaction mixture was centrifuged, and an aliquot of the supernatant solution was assayed for GGT activity by CE-TOF/MS.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Alignment of the translated sequence of the Arabidopsis OXPT gene and protein sequences of S0Pase from animals.

**Supplemental Figure S2.** A14g25720 (QCT) knockout mutants.

**Supplemental Figure S3.** Detection of S0P using HPLC.

**Supplemental Table S1.** γ-Glutamyl transferase activity in the wild type and ggt mutants.

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