1 **Characterization of γ -Glutamyl Peptidases and γ -Glutamyl Cyclotransferases for**
2 **Glutathione Degradation in *Arabidopsis***

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30 **Summary**

- 31 • Organic sulfur is stored as glutathione (GSH) in plants. In *Arabidopsis*, γ -glutamyl
32 cyclotransferases (GGCT2;1, GGCT2;2, and GGCT2;3) degrade cytosolic GSH,
33 but they do not fully explain the rapid GSH turnover. Here, we demonstrate that
34 γ -glutamyl peptidases, GGP1 and GGP3, play a substantial role in degrading GSH
35 in the cytosol.
- 36 • We conducted yeast complementation assay and activity assay of recombinant
37 proteins to identify the novel GSH degradation enzymes. The expression patterns
38 were investigated by RT-qPCR. GSH concentrations in the mutants were also
39 analyzed.
- 40 • *GGP1* complemented the yeast phenotype. Recombinant GGP1 and GGP3 showed
41 reasonable K_m values considering cytosolic GSH concentration, and their activity
42 was comparable to that of GGCTs. The *GGP1* transcript was highly abundant in
43 mature organs such as rosette leaves. The expression of *GGCT2;1* was
44 conspicuously enhanced under sulfur deficiency. GSH concentration was higher in
45 *ggp1* knockout mutants regardless of nutritional conditions; the concentration was
46 higher in *ggct2;1* knockout mutants under sulfur-deficient conditions.
- 47 • We propose a model wherein cytosolic GSH is degraded fundamentally by GGP1.
48 The degradation is accelerated by GGCT2;1 under sulfur deficiency. Given the
49 energy cost throughout the reactions, GGPs could render a more efficient route for
50 GSH degradation than GGCTs.

51

52 **Key words:** *Arabidopsis thaliana*, cysteine, gamma-glutamyl cycle, gamma-glutamyl
53 cyclotransferase, gamma-glutamyl peptidase, glutathione metabolism, sulfur deficiency,

54 sulfur metabolism

55

56 **Introduction**

57 Glutathione (GSH; L- γ -glutamyl-L-cysteinyl-glycine) plays a variety of
58 essential roles in plants. GSH reversibly undergoes redox reactions through its thiol
59 residue and maintains the redox status of cells. When oxidized, two glutathione
60 molecules form dimer and become oxidized glutathione (GSSG), which is returned to
61 the reduced GSH by glutathione reductase. GSH is essential in alleviating oxidative
62 stress in plants since most GSH in the cell is in its reduced form and can reduce
63 oxidants (Foyer and Noctor, 2011; Noctor *et al.*, 2011). GSH is also involved in the
64 redox regulation of proteins (Gao *et al.*, 2009). Phytochelatins, the polymerized forms
65 of GSH, contribute to plant tolerance toward heavy metals and metalloids, such as
66 cadmium and arsenate (Cobbet, 2000; Mendoza-Cózatl, 2011). GSH also plays another
67 role in the storage of reduced sulfur in cells. Sulfur is taken up by plant cells mainly as
68 sulfate, which is then reduced to sulfide. Sulfide can be incorporated into the amino acid
69 skeleton of *O*-acetyl-L-Ser to produce Cys, which is the donor for all further
70 metabolites (Koprivova and Kopriva, 2014). Cys, however, is not a storage form of
71 reduced sulfur because extra concentration of cysteine is toxic due to its high reactivity
72 (Romero *et al.*, 2014 ; Deshpande *et al.*, 2017). Cys is incorporated into GSH, which
73 exists in high concentrations in plant tissues, acts as a substrate for central biochemicals,
74 and maintains Cys availability for protein synthesis. Thus, GSH acts as a precursor for
75 metabolites that, in turn, may be related to the GSH degradation rate (Leustek *et al.*,
76 2000). GSH concentrations in the phloem sap are in the mM range, and GSH is
77 considered the transport form of reduced sulfur (Kuzuhara *et al.*, 2000).

78 GSH is synthesized from its constituent amino acids via a two-step
 79 ATP-dependent reaction. First, γ -glutamylcysteine (γ -EC) is synthesized from Glu and
 80 Cys by γ -EC synthetase (Hell and Bergmann, 1990), which is encoded by *GSH1* (May
 81 and Leaver, 1994), and Gly is incorporated by GSH synthetase encoded by *GSH2*
 82 (Wang and Oliver, 1996). However, GSH degradation pathways are not as
 83 straightforward as this synthesis pathway. In mammals, GSH is degraded during the
 84 γ -glutamyl cycle (Supporting Information Fig. S1; Orłowski and Meister, 1970; Meister
 85 and Larsson, 1995). In this cycle, GSH is degraded outside cells by an exoenzyme,
 86 γ -glutamyl transpeptidase (GGT), which hydrolyzes GSH and transfers the Glu residue
 87 to water or other amino acids. When transferred to water, Glu is produced; when
 88 transferred to amino acids, γ -glutamyl amino acids are produced. Glu and γ -glutamyl
 89 amino acids are incorporated into cells, and γ -glutamyl amino acids are processed by
 90 cytosolic enzyme, γ -glutamyl cyclotransferase (GGCT). This enzyme detaches the
 91 γ -glutamyl residue of γ -glutamyl amino acids, and releases it as 5-oxoproline.
 92 5-oxoproline can be converted to Glu by another cytosolic enzyme, oxoprolinase. The
 93 other reaction product of GSH degradation by GGT is cysteinylglycine (Cys-Gly). This
 94 dipeptide is degraded into Cys and Gly by dipeptidases. In this way, GSH is degraded
 95 into three constituent amino acids, Glu, Cys, and Gly, and they are utilized for
 96 biosynthesis of proteins or GSH in the cells (Fig. S1).

97 In *Arabidopsis*, the turnover of GSH is as high as 80% in 1 d under normal
 98 conditions (Ohkama-Ohtsu *et al.*, 2008), and genes responsible for the degradation
 99 pathway have been extensively explored. There are four homologs of the mammalian
 100 GGTs: GGT1, 2, 3, and 4. GGT1 and GGT2 are localized to the apoplast and GGT4 to
 101 the vacuole (Grzam *et al.*, 2007; Martin *et al.*, 2007; Ohkama-Ohtsu *et al.*, 2007a, b).

102 Among them, GGT1 and GGT4 are actively expressed in the whole plant body, so a
 103 *ggt1/ggt4* knockout mutant, which shows no GGT activity, was analyzed in detail
 104 (Ohkama-Ohtsu *et al.*, 2008). However, GSH degradation rate and GSH concentration
 105 were not affected by the defect in GGT activity. This strongly suggested that GGT is not
 106 involved in GSH degradation; instead, GGP1 is responsible for GSSG degradation
 107 (Ohkama-Ohtsu *et al.*, 2007a) and GGT4 is for GSH-conjugate degradation (Grzam *et*
 108 *al.*, 2007; Ohkama-Ohtsu *et al.*, 2007b). After all, other GSH degradation pathways
 109 needed to be identified.

110 Ohkama-Ohtsu *et al.* (2008) also found that 5-oxoproline levels are correlated
 111 with GSH or γ -EC levels, and proposed that GGCT, a cytosolic enzyme which degrades
 112 γ -glutamyl amino acids and releases 5-oxoproline, also degrades GSH. In addition,
 113 activity assay using protein extract suggested that 5-oxoproline is more likely to be
 114 supplied by GSH degradation than by γ -EC degradation (Ohkama-Ohtsu *et al.*, 2008).
 115 Later, GGCT which has GSH degradation activity was indeed identified in mammals
 116 (Kumar *et al.*, 2012), and three homologs were identified in *Arabidopsis*: *GGCT2;1*,
 117 *GGCT2;2*, and *GGCT2;3*. All of them degrade physiological concentration of GSH into
 118 5-oxoproline and Cys-Gly *in vitro* and complement the GSH degradation-defective
 119 yeast mutant (Paulose *et al.*, 2013; Kumar *et al.*, 2015). Among these *GGCT* genes,
 120 expression patterns of *GGCT2;1* have been analyzed in detail. *GGCT2;1* mRNA is
 121 rapidly accumulated under various conditions: pollen tube growth (Want *et al.*, 2008),
 122 heavy metal stress (Kovalchuk *et al.*, 2005), salinity stress (Gong *et al.*, 2005), and
 123 sulfur starvation (Maruyama-Nakashita *et al.*, 2005; Hubberten *et al.*, 2012; Bielecka *et*
 124 *al.*, 2014). Paulose *et al.* (2013) reported that the promoted *GGCT2;1* expression under
 125 arsenite stress can contribute to heavy metal tolerance by recycling Glu in GSH and

126 saving the energy for *de novo* Glu synthesis. Joshi *et al.* (2019) also reported that
 127 GGCT2;1 is involved in the regulation of root architecture under sulfur deficiency by
 128 recycling Cys in GSH. However, what is responsible for the rapid turnover of GSH is
 129 still unclear because the expression of *GGCT2;1* is very low under normal conditions.
 130 We hypothesized that enzymes other than GGCT2;1 contribute to cytosolic GSH
 131 degradation under normal conditions.

132 In the present study, we show that the cytosolic enzymes γ -glutamyl peptidase
 133 1 (GGP1) and GGP3 have GSH degradation activity comparable to that of GGCT2;1,
 134 GGCT2;2, and GGCT2;3. Because GGP1 and GGP3 also process GSH-conjugates in
 135 the biosynthesis of glucosinolates and camalexin (Geu-Flores *et al.*, 2009), they are
 136 considered bifunctional enzymes involved in GSH degradation and glucosinolates and
 137 camalexin synthesis. We further suggest that cytosolic GSH is fundamentally degraded
 138 by GGP1, except that GGCT2;2 is crucial in developing organs and GGCT2;1 is vital
 139 under sulfur deficiency.

140

141 **Materials and Methods**

142 **Yeast strains and screening**

143 The *Saccharomyces cerevisiae* strains used in this study, listed in Table S1,
 144 were obtained from EUROSCARF (<http://www.euroscarf.de/>). Strains Y05729 (*dug2Δ*)
 145 and Y05729 (*dug3Δ*) were constructed from BY4741 by insertional mutagenesis
 146 (Winzeler *et al.*, 1999). All strains were missing *MET15* and were unable to assimilate
 147 inorganic sulfur.

148 An *A. thaliana* cDNA library was cloned into the expression plasmid pFL61
 149 and transformed into competent yeast cells of *dug2Δ* or *dug3Δ* strains (Minet *et al.*,

150 1992) using the lithium acetate method (Ito *et al.*, 1983). The transformants were
151 screened on minimal selection plates (SD-ura), in which all sulfate ions were replaced
152 with chloride ions and supplemented with 200 μ M GSH. All plates were incubated at
153 28 °C for 3–5 d. The transformation efficiency was calculated from yeast grown on
154 plates (SD-ura) supplemented with 200 μ M methionine. The plasmids transformed into
155 yeast were extracted using Zymoprep™ Yeast Plasmid Miniprep I (Zymoresearch,
156 <http://www.zymoresearch.com/>).

157 The inserts in the clones were sequenced with the ABI PRISM 3130 and 3500
158 Genetic Analyzer (Applied Biosystems), and primers pGK5 and pGK3 listed in Table
159 **S2** (Nozawa *et al.*, 2006).

160

161 **Construction of the plasmids for transformation into yeast**

162 Total RNA was extracted from *A. thaliana* using an RNeasy Plant Mini Kit
163 (Qiagen, Hilden, Germany) followed by cDNA synthesis with SuperScript III reverse
164 transcriptase (Invitrogen, <https://www.thermofisher.com/>) and the Oligo (dT)₁₆ primer.
165 The ORF sequences for *GGP1* (AT4G30530), *GGCT2;1* (AT5G26220), *GGCT2;2*
166 (*AT4G31290*), and *GGCT2;3* (*AT1G44790*) were amplified from the *A. thaliana* cDNA
167 template using the primer sets listed in Table **S2**. The amplified product for *GGP1* was
168 subcloned into the *EcoRI*-*XhoI* site, and those for *GGCT2;1*, *GGCT2;2*, and *GGCT2;3*
169 were subcloned into the *BamHI*-*EcoRI* site of the p416TEF yeast expression vector
170 (Mumberg *et al.*, 1995). These plasmids were transformed into *dug2Δ* and *dug3Δ* yeast
171 strains using the lithium acetate method (Ito *et al.*, 1983).

172

173 **Construction of *E. coli* expression strains**

174 The coding sequences (CDSs) of *GGP1*, *GGP3*(*AT4G30550*), *GGCT2;1*,
 175 *GGCT2;2* and *GGCT2;3* were obtained from the *Arabidopsis* Information Resource
 176 (<https://www.arabidopsis.org/>), and they were amplified by PCR using KOD-Plus-Neo
 177 (TOYOBO, Osaka, Japan) with the primers listed in Table **S3**. The PCR products were
 178 cloned into the entry vector pENTR/D-TOPO (Invitrogen, MA, USA), and the
 179 sequences of inserts were confirmed by Eurofins Genomics (Tokyo, Japan) with M13
 180 primers (Table **S3**). Subsequently, each CDS was cloned into the destination vector
 181 pDEST17 (Invitrogen, MA, USA) via the LR recombination reaction. Finally, the
 182 constructed plasmids were transformed into *E. coli* expression strain BL21-AI
 183 (Invitrogen, MA, USA). All operations were performed according to the manufacturers'
 184 protocols.

186 **Expression and purification of the recombinant proteins**

187 BL21-AI possessing each construct was cultured in LB medium containing 100
 188 mg/ml ampicillin up to O.D.₆₀₀ ~ 0.6 at 37 °C at 150 rpm. Then, L-arabinose was added
 189 to a final concentration of 0.02% (w/v), and the cells were cultured for another 17 h at
 190 18 °C at 120 rpm. Cells were harvested by centrifugation at 2500 × *g* for 5 min at 4 °C.
 191 The target proteins were obtained as N-terminal His-tagged proteins, followed by
 192 purification via His60 Ni Gravity Column Purification Kit (Clontech Laboratories, CA,
 193 USA), according to the manufacturer's instructions; except that 1 mM
 194 phenylmethanesulfonyl fluoride (PMSF) was added to the extraction and wash buffers
 195 of the kit. Out of the five proteins expressed in *E. coli*, *GGP1*, *GGP3*, and *GGCT2;3*
 196 were collected from the soluble fraction and utilized for the subsequent experiments.
 197 The proteins were dialyzed overnight in a buffer containing 50 mM Tris-HCl (pH 8.0)

198 and 150 mM NaCl at 4 °C. The results were confirmed using SDS-PAGE (Fig. S2).

199 Protein concentrations were determined using the Bradford reagent (Bio-Rad, CA,
200 USA) and bovine serum albumin as the standard.

201

202 **Activity assay of recombinant proteins for GSH or other γ -glutamyl compounds**

203 Kinetic parameters were determined using 0.5 μ g of GGP1, 0.25 μ g of GGP3,
204 or 2.2 μ g of GGCT2;3. The proteins were incubated in 50 μ l of reaction mixture
205 containing 50 mM Tris-HCl (pH 8.0) and 0.25 mM to 15.0 mM GSH for 30 min at
206 37 °C, and the reaction was terminated by adding 10 μ l of 1.5 M HCl. The solution was
207 centrifuged at 11,000 $\times$ g for 15 min at 4 °C, and the Cys-Gly released in the
208 supernatant was quantified by HPLC as described below. Kinetic parameters were
209 calculated using GraphPad Prism 9 software (GraphPad Software, CA, USA). For
210 degradation activity for other γ -glutamyl compounds, 0.5 μ g of GGP1 or 0.25 μ g of
211 GGP3 was incubated in 50 μ l of reaction mixture containing 50 mM Tris-HCl (pH 8.0)
212 and 5.0 mM GSH, 5.0 mM γ -Glu-Cys (Sigma-Aldrich), 5.0 mM α -Glu-Ala
213 (Sigma-Aldrich), or 2.5 mM GSSG. The reaction was conducted as described previously.
214 As described below, released Cys-Gly was quantified for GSH, Cys was quantified for
215 γ -Glu-Cys, and Ala was quantified for γ -Glu-Ala.

216

217 **Analysis of Ala**

218 Ala in the reaction solutions of activity assays was derivatized with the
219 AccQ-Fluor Reagent Kit (Waters, <http://www.waters.com/waters/home>) and measured
220 by HPLC using an AccQ-Tag column (Waters) according to the manufacturer's
221 instructions.

222

223 **Thiol analysis with HPLC**

224 Thiols extracted from plant tissues with 0.01 N HCl, or in the reaction solutions
225 of activity assays, were quantified by HPLC after derivatization with
226 monobromobimane (Minocha *et al.*, 2008; Nishida *et al.* 2016). The HPLC system
227 (Shimadzu, Japan) consisted of a system controller (CBM-20A), two pumps
228 (LC-20AD), a degasser (DGU-20A₃), an autosampler (SIL-20A), a column oven
229 (CTO-20AC), and a fluorescence detector (RF-20A). The column was a Shim-pack
230 FC-ODS (4.6 mm × 150 mm; Shimadzu, Japan).

231

232 **Hydroponic culture of plants for sampling at vegetative and reproductive stages**

233 *Arabidopsis thaliana* Col-0 wild-type plants were grown hydroponically in 50
234 ml conical tubes at 22 °C under 16 h light ($100 \mu\text{mol}^{-1}\text{m}^{-2}\text{s}^{-1}$) / 8 h dark as described by
235 Ohkama-Ohtsu *et al.* (2007b) using MGRL medium (Fujiwara *et al.*, 1992; Hirai *et al.*,
236 1995). The vegetative-stage samples were grown for 22 d, three oldest leaves from the
237 first true leaf were collected as mature leaves, and three youngest leaves were collected
238 as young leaves. For the reproductive stage, samples were grown for 33 d, and flower +
239 buds, siliques 1–2 cm in length, stems, and all cauline and rosette leaves were collected
240 separately. Roots were divided into three equally long parts, and the upper, middle and
241 lower roots were collected separately. Samples were immediately frozen with liquid
242 nitrogen and stored at -80 °C until RNA extraction.

243

244 **Liquid culture of plants in flasks**

245 *Arabidopsis* plants were grown in flasks, as described in Xiang and Oliver

(1998) using MGRL medium (Fujiwara *et al.*, 1992). For sulfur-deficient treatments, all sulfate ions in the MGRL medium were replaced with an equal molar concentration of chloride ions (Hirai *et al.*, 1995). In nitrogen-deficient treatments, all the nitrate ions were replaced with an equal molar concentration of chloride ions. In the nitrogen-deficient experiment, 12 d-old plants grown in a standard medium were transferred to a nitrogen-deficient medium and grown for an additional 2 d. In sulfur-deficient experiments, 10 d-old plants grown in the standard medium were transferred to sulfur-deficient medium and grown for an additional 4 d. Control plants were grown for 14 d in a standard MGRL medium, and the medium was replaced after 10 d. The entire bodies of plants in each flask were pooled immediately frozen with liquid nitrogen and stored at -80 °C until analysis.

RNA extraction and quantitative PCR analysis

RNA was extracted from frozen samples using the RNeasy Plant Mini Kit (Qiagen, Germany), followed by reverse transcription using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Bio, Shiga, Japan). Quantitative PCR was conducted using the KAPA SYBR FAST qPCR Master Mix (2X) Kit (KAPA Biosystems, MA, USA) and LightCycler 96 (Roche Diagnostics, Switzerland) following the manufacturer's protocols. The absolute quantification method was adopted using serial dilutions of DNA with known copy numbers for standard curves. The copy number of *β-tubulin* (*TUB4*, AT5G44340) was determined as the internal standard. The primers used are listed in Table S4. The normalized copy number was determined by dividing copy number of each gene by that of *β-tubulin*.

270 **Screening of *Arabidopsis* T-DNA knockout mutants for *ggp1* and *ggct2;1***

271 Unless otherwise indicated, *Arabidopsis thaliana* plants were grown on
272 rockwool in a plastic container at 22 °C under a 24 h photoperiod (150 $\mu\text{mol m}^{-2} \text{s}^{-1}$)
273 under 16 h light/8 h dark conditions to establish mutant lines honoring T-DNA
274 homozygously. The primers used for screening *ggp1* and *ggct2;1* mutant plants are
275 shown in Table S5.

276 The *ggp1-1* mutant, which was used by Geu-Flores *et al.* (2011), was provided
277 by GABI-kat (<http://www.gabi-kat.de/>, stock no. GK-319F10) (Rosso *et al.*, 2003).
278 Homozygous plants were screened by PCR using the gene-specific primers GK-319-F,
279 GK-319-R and the T-DNA left-border primer GABI-8474 (<http://www.gabi-kat.de/>). A
280 lack of amplifiable transcripts of *At4g30530* in the *ggp1-1* mutant was reported by
281 Geu-Flores *et al.* (2011), and we also confirmed by quantitative RT-PCR using primers
282 *At4g30530F* and *At4g30530R*. The *ggp1-2* mutant (SALK_089634) was provided by
283 the Salk Institute (Alonso *et al.*, 2003) and was obtained from the *Arabidopsis*
284 Biological Resource Center (ABRC, <https://abrc.osu.edu/>). Homozygous plants were
285 screened using the gene-specific primers SALK_089634F, SALK_089634R, and the
286 T-DNA left-border primer pROK α 3 (Lin and Oliver, 2008). The lack of an amplifiable
287 transcript of *At4g30530* in the *ggp1-1* mutant was confirmed by quantitative RT-PCR
288 using primers *At4g30530F* and *At4g30530R*.

289 The *ggct2;1-2* mutant (SALK_56007), which was used by Joshi *et al.* (2019),
290 was provided by the Salk Institute (Alonso *et al.*, 2003) and was obtained from the
291 *Arabidopsis* Biological Resource Center (ABRC, <https://abrc.osu.edu/>). Homozygous
292 plants were screened by PCR using the gene-specific primers GGCT2,1F, GGCT2,1R,
293 and the T-DNA left-border primer pROK α 3 (Lin and Oliver, 2008). A lack of an

294 amplifiable transcript of *At5g26220* in the *ggct2;1-2* mutant was reported by Joshi *et al.*
295 (2019), and we also confirmed by RT-PCR using the primers At5g26220F and
296 At5g26220R.

297

298 **Results**

299 **Isolation of *A. thaliana* cDNA clones that complement yeast *dug2Δ* or *dug3Δ*** 300 **mutants**

301 An *A. thaliana* cDNA library built in the yeast expression vector pFL61 (Minet
302 *et al.*, 1992) was transformed into the *Saccharomyces cerevisiae* yeast mutants *dug2Δ* or
303 *dug3Δ* (DUG: Defective in Utilization of GSH; Ganguli *et al.*, 2007). In *Saccharomyces*
304 *cerevisiae*, three mutants, *dug1Δ*, *dug2Δ*, and *dug3Δ*, are known to be defective in GSH
305 utilization (Ganguli *et al.*, 2007). (DUG2-DUG3)₂ complex first degrades GSH into Glu
306 and Cys-Gly, and then the (DUG1)₂ homodimer degrades Cys-Gly into Cys and Gly
307 (Kaur *et al.*, 2009, 2012). As a result of this transformation, *c.* 4.6×10^4 yeast
308 transformants were obtained in *dug2Δ*, and two independent colonies were isolated from
309 the minimal selection plates with GSH as the sole sulfur source. From the *c.* 6.9×10^4
310 yeast transformants in *dug3Δ*, three independent colonies were isolated under the same
311 growth conditions. Plasmids were isolated from these colonies, and cDNAs expressed
312 from the library were sequenced. All clones that complemented the *dug2Δ* and *dug3Δ*
313 yeast mutants corresponded to the gene *γ-glutamyl peptidase 1 (GGP1)*, which was
314 initially identified as an enzyme processing GSH-conjugates in the biosynthesis of
315 glucosinolates and camalexin (Geu-Flores *et al.*, 2009).

316 To confirm the complementation of *dug2Δ* and *dug3Δ* yeast with *GGP1*, the
317 open reading frame (ORF) of *GGP1* was cloned into the yeast expression vector

318 p416TEF (Mumberg *et al.*, 1995) and transformed into *dug2Δ* and *dug3Δ* mutants. Both
 319 yeast mutants transformed with the *GGP1* ORF grew on the medium when GSH was
 320 the sole sulfur source (Fig. 1). This result suggested that GGP1 from *Arabidopsis* is
 321 sufficient to degrade GSH, whereas yeast DUG2 and DUG3 need to form an active
 322 complex. The genes *GGCT2;1*, *GGCT2;2*, and *GGCT2;3* were also cloned into
 323 p416TEF and were introduced into *dug2Δ* or *dug3Δ* mutants. As previously
 324 demonstrated, all GGCT genes complemented both yeast mutants (Fig. 1) (Kumar *et al.*,
 325 2015).

326

327 **Degradation activities of recombinant proteins from *E. coli***

328 We investigated the enzymatic properties of GGPs and GGCTs using
 329 recombinant proteins. There are five *GGP* genes in *Arabidopsis*, but the expression of
 330 *GGP2*, *GGP4*, and *GGP5* is very low, and only *GGP1* and *GGP3* are actively expressed
 331 across all tissues (Geu-Flores *et al.*, 2011); therefore, in addition to GGP1, GGP3 was
 332 analyzed in this experiment.

333 GGP1, GGP3, GGCT2;1, GGCT2;2, and GGCT2;3 were expressed in *E. coli*.
 334 GGP1, GGP3, and GGCT2;3 collected from the soluble fraction were utilized for the
 335 subsequent measurement of enzyme kinetics, and analyzed together with the reported
 336 properties of GGCT2;1 and GGCT2;2 (Paulose *et al.*, 2013; Kumar *et al.*, 2015). The
 337 activity and affinity of the proteins were analyzed with various GSH concentrations
 338 ranging from 0.25 mM to 15.0 mM (Fig. S3; Table 1). The K_m of GGP1, GGP3, and
 339 GGCT2;3 was 5.0 mM (GGP1), 3.7 mM (GGP3), and 6.7 mM (GGCT2;3),
 340 respectively; their k_{cat} was 19.9 min⁻¹ (GGP1), 70.3 min⁻¹ (GGP3), and 18.5 min⁻¹
 341 (GGCT2;3), respectively.

342 In addition to that toward GSH, degradation activity of these enzymes toward
343 other γ -glutamyl compounds—namely, oxidized glutathione (GSSG),
344 γ -glutamyl-cysteine (γ -Glu-Cys), and γ -glutamyl-alanine (γ -Glu-Ala)—was also
345 analyzed (Fig. **S4**). However, the activity of GGCT2;3 toward γ -Glu-Cys and γ -Glu-Ala
346 was excluded from the analysis because it is known to be much lower than that toward
347 GSH (Kumar *et al.*, 2015). The results showed that GGP1, GGP3, and GGCT2;3 hardly
348 degraded γ -Glu-Cys, γ -Glu-Ala, or GSSG.

349

350 **Comparison of expression levels of *GGP* and *GGCT* genes among organs**

351 The distribution of *GGP1*, *GGP3*, *GGCT2;1*, *GGCT2;2*, and *GGCT2;3* was
352 clarified by dividing plants at the vegetative and reproductive stage into 10 parts and
353 analyzing the expression levels of these genes by quantitative RT-PCR using the
354 absolute quantification method. The components of the 10 parts were the mature and
355 young leaves of vegetative-stage plants and the flower/buds, siliques, stems, cauline and
356 rosette leaves, and the upper, middle, and lower roots of reproductive-stage plants. In
357 general, all *GGP* and *GGCT* genes, except *GGCT2;1*, were constitutively expressed in
358 all organs (Fig. **2**). In particular, *GGP1* transcripts in rosette leaves were abundant. In
359 contrast, the expression of *GGCT2;1* was much lower than that of other four genes in all
360 organs.

361 There was also a notable trait that the amount of *GGP3* transcript was
362 significantly higher in mature leaves than in young leaves at the vegetative stage (Fig.
363 **2a**), and a similar tendency was observed for *GGP1* ($P < 0.1$). As mentioned above, the
364 *GGP1* transcript was highly abundant when the leaves achieved a rosette arrangement at
365 the reproductive stage (Fig. **2b,c**). In contrast, the amount of *GGCT2;2* transcript was

significantly higher in young leaves than mature ones at the vegetative stage (Fig. **2a**).
A similar trait was confirmed for the reproductive stage wherein the *GGCT2;2*
transcript in cauline leaves was significantly higher than that in rosette leaves (Fig. **2c**).
Furthermore, the *GGCT2;2* transcript in the lower roots, which is considered as the
youngest in roots, was significantly larger than in the middle and upper roots (Fig. **2d**).

371

372 **Changes in expression of *GGP* and *GGCT* genes under sulfur and nitrogen** 373 **deficiency**

We also investigated the expression of *GGP1*, *GGP3*, *GGCT2;1*, *GGCT2;2*
and *GGCT2;3* under sulfur or nitrogen deficiency because GSH functions in the storage
of organic sulfur and nitrogen, and in fact, the expression of *GGCT2;1* sharply increases
under sulfur deficiency (Joshi *et al.*, 2019). The changes in expression of *GGP1*, *GGP3*,
GGCT2;1, *GGCT2;2* and *GGCT2;3* in wild-type plants under nitrogen or sulfur
deficiency are shown in Fig. **3**. The mRNA level of *GGCT2;1* was drastically increased
under sulfur deficiency by 31-fold compared with that under the control condition and
that of *GGCT2;2* was also significantly increased by 2.3-fold.

382

383 **GSH accumulation in the *gpp1* and *ggct2;1* knockout mutants**

To confirm the contribution of *GGP1* and *GGCT2;1* to GSH degradation in
planta, GSH concentration in the *gpp1-1* knockout mutant (GK-319F10, Geu-Flores *et*
al., 2011), *ggct2;1-2* knockout mutant (SALK_56007, Joshi *et al.*, 2019), and their
corresponding wild-type plants (Columbia-0) was measured using 14 d-old liquid
cultured seedlings. Under normal conditions, GSH concentration in *gpp1-1* was
significantly higher than that in the wild-type plants, whereas *ggct2;1-2* did not show

any significant difference (Fig. 4), suggesting that GGP1 degrades GSH under normal conditions.

We also measured GSH concentrations in mutants under sulfur- or nitrogen-deficient conditions. Under sulfur deficiency, the *ggp1-1* and *ggct2;1-2* mutants showed significantly higher GSH levels than the wild-type plants (Fig. 4). Higher GSH levels under sulfur deficiency have also been reported for *ggct2;1-1* (SALK_117578), another T-DNA insertion mutant of *GGCT2;1* (Joshi *et al.*, 2019). However, under nitrogen deficiency, the elevation of GSH levels was only observed in *ggp1-1* and not in *ggct2;1-2*, which was similar to normal conditions. GSH accumulation by the deletion of *GGP1* was also confirmed for *ggp1-2* (SALK_089634), another T-DNA insertion mutant of *GGP1*, under normal and sulfur- and nitrogen-deficient conditions (Fig. S5). These results suggest that GGP1 constantly degrades GSH, and *GGCT2;1* also breaks it down under sulfur deficiency.

mRNA levels of *GGP* and *GGCT* genes in wild-type, *ggp1-1*, and *ggct2;1-2* mutant plants under the control condition, sulfur or nitrogen deficiency

Homologous proteins often complement the lack of protein function. Therefore, the expression of other *GGP* and *GGCT* genes in *ggp1-1* and *ggct2;1-2*, as well as in wild-type plants, was investigated to determine whether this is the case for GGP1 or *GGCT2;1*, (Fig. 5). The mRNA level of *GGP3* increased in the *ggp1-1* mutant plants compared with that in wild-type plants under sulfur deficiency (Fig. 5b), suggesting a possibility that GGP3 functionally complements GGP1 under these conditions. The mRNA level of *GGCT2;2* increased in *ggct2;1-2* mutant plants compared with that in wild-type plants under sulfur deficiency, suggesting a possibility that *GGCT2;2*

414 functionally complements GGCT2;1 under these conditions.

415

416 Discussion

417 Newly discovered GSH degradation activity of GGP1 and GGP3

418 In the present study, we found that GGPs, enzymes that were originally
 419 reported to process GSH-conjugates (Geu-Flores *et al.*, 2009), also degrade GSH. The
 420 yeast mutants defective in GSH degradation—*dug2Δ* or *dug3Δ*—were complemented by
 421 *Arabidopsis* *GGP1* (Fig. 1) and grew on the medium where GSH was the sole sulfur
 422 source. In addition, the recombinant GGP1 and GGP3 proteins showed activity for GSH
 423 degradation (Fig. S3; Table 1). The K_m values of GGP1 and GGP3 for GSH were 5.0
 424 mM and 3.7 mM, which are reasonable because cytosolic GSH concentration ranges
 425 from 2.8 mM to 4.5 mM (Koffer *et al.*, 2013). This reasonability is also confirmed by
 426 the fact that the K_m values of GGCTs are 1.9 mM for GGCT2;1 (Paulose *et al.*, 2013),
 427 1.7 mM for GGCT2;2 (Kumar *et al.*, 2015), and 6.7 mM (Table 1) or 4.9 mM (Kumar *et*
 428 *al.*, 2015) for GGCT2;3. The activity of GGP1 and GGP3 was moderate because their
 429 k_{cat} values were 1.1-fold and 3.8-fold higher than that of GGCT2;3 (Table 1),
 430 respectively, whereas the k_{cat} value of GGCT2;2 is 5.7-fold higher than that of
 431 GGCT2;3 (Kumar *et al.*, 2015).

432 Another remarkable trait of GGPs is the abundance of *GGP1* transcripts. The
 433 *GGP1* transcript was the largest among *GGPs* and *GGCTs* in the reproductive stage
 434 rosette leaves (Fig. 2), which occupied the highest volume in the plant body. The *GGP1*
 435 transcript was also the most abundant in 2-week-old liquid cultured plants (Fig. 5).
 436 Additionally, in the TAIR database (<http://www.arabidopsis.org/>), the number of
 437 expressed sequence tags (ESTs) associated with GGP1, GGP3, GGCT2;1, GGCT2;2

438 and GGCT2;3 were 335, 66, 13, 118, and 52, respectively, and five clones that
439 complemented the *dug2Δ* or *dug3Δ* mutants in our screening all corresponded to *GGP1*.

440 Considering these facts, it is highly possible that GGPs, especially GGP1,
441 substantially contribute to GSH degradation *in planta*. The *gfp1* knockout mutant
442 significantly accumulated GSH compared with the wild-type plants (Fig. 4). Therefore,
443 GGP1 and GGP3, in addition to GGCT2;1, GGCT2;2, and GGCT2;3, should be
444 considered when examining GSH degradation in the cytosol. In plants, the turnover rate
445 of GSH is so fast that 80% of GSH is degraded in 1 d, and a large portion of this
446 degradation is estimated to be cytosolic (Ohkama-Ohtsu *et al.*, 2008). It is a future
447 challenge to investigate whether GGPs and GGCTs can explain this high degradation
448 rate.

449 It should also be noted that GSH degradation by GGPs is reasonable in terms
450 of energy efficiency. This is because the pathway through GGPs does not require ATP
451 for Glu production from GSH (Geu-Flores *et al.*, 2011), whereas the pathway through
452 GGCTs requires ATP to convert 5-oxoproline to Glu (Ohkama-Ohtsu *et al.*, 2008). Thus,
453 it is considered reasonable for plants to use GGPs when degrading GSH.

454

455 **Bifunctionality of GGP1 and GGP3 in GSH degradation and glucosinolate** 456 **synthesis**

457 GGP1 and GGP3 were initially reported as enzymes that process
458 GSH-conjugates in the biosynthesis of glucosinolates and camalexin (Geu-Flores *et al.*,
459 2009); therefore, the competition between the reactions can be considered. The K_m value
460 of GGP1 was 5.0 mM for GSH (Table 1), which is appropriate considering the cytosolic
461 GSH concentration mentioned above. In contrast, the K_m value for a GSH-conjugate

462 *S*-[(*Z*)-phenylaceto-hydroximoyl]-L-glutathione (GS-B) is 37 μ M, which also suggests
 463 that GS-B can be a physiological substrate (Geu-Flores *et al.*, 2009). Therefore, both
 464 reactions are considered to occur in plants, competing with each other. The ATTED-II
 465 database (Obayashi *et al.*, 2018, <https://atted.jp/>) shows that *GGP1* is coregulated with
 466 genes involved in glucosinolate synthesis pathways and the sulfur assimilation pathway,
 467 such as *APS1* (*ATP sulfurylase 1*, At3g22890), *APR1* (*APS reductase 1*, AT4G04610),
 468 *APK1* (*APS kinase 1*, AT2G14750), *APK2* (*APS kinase 2*, At4g39940), *APS3* (*ATP*
 469 *sulfurylase 3*, AT4G14680), and *SERAT2;2* (*serine acetyltransferase2;2*, At3g13110).
 470 Because GSH is the primary storage form of organic sulfur in cells, it is conceivable
 471 that GSH degradation, together with sulfur assimilation, supplies organic sulfur when
 472 the demand is high.

473 If GGPs process both GSH and GSH-conjugates, the priority of the reactions
 474 likely varies depending on the substrate concentration and is considered reasonable in
 475 plants. The affinity of GGPs for GSH-conjugates is much higher than for GSH; when
 476 GSH-conjugates accumulate, it is likely that processing GSH-conjugates will be
 477 prioritized over GSH degradation, leading to an automatic reduction in GSH
 478 degradation. Although it is well known that glucosinolates and camalexin function in
 479 alleviating biotic stresses, high concentrations of GSH are also crucial for resistance to
 480 viruses, bacteria, and fungi because GSH functions in ROS scavenging and signaling
 481 (Hernández *et al.*, 2017). Therefore, it is very reasonable for plants experiencing biotic
 482 stress to synthesize glucosinolates and camalexin and simultaneously decrease GSH
 483 degradation. Additionally, it is notable that *Ralstonia solanacearum*, a pathogenic
 484 bacterium, produces the virulence effector protein RipAY, which has GGCT activity
 485 (Mukaihara *et al.*, 2016). GSH degradation by RipAY causes the depletion of the GSH

486 pool in plant cells, increasing susceptibility in the early stages of infection.

487 In contrast, GSH degradation may be prioritized over GSH-conjugate
488 degradation under sulfur starvation because the transcript levels of upstream genes in
489 glucosinolate synthesis pathways are downregulated under sulfur-deficient conditions
490 (Aarabi *et al.*, 2016), and thus, the concentrations of GSH-conjugates are presumed to
491 decrease. It is reasonable that GSH degradation activates under sulfur deficiency
492 because GSH degradation functions as the distribution of stored organic sulfur. In
493 addition, it has recently been shown that glucosinolates are degraded under sulfur
494 deficiency to reallocate sulfur atoms in them, and that GSH is required for the
495 glucosinolate degradation pathway (Sugiyama *et al.*, 2021). Therefore, glucosinolate
496 synthesis by GGPs, which consumes GSH, may be inactivated under sulfur deficiency,
497 and GSH is possibly utilized for glucosinolate degradation. Overall, bifunctionality of
498 GGPs potentially contribute to sulfur flow shift under sulfur deficiency from sulfur
499 consumption in glucosinolate synthesis to sulfur reallocation through GSH and
500 glucosinolate degradation.

501 Incidentally, *GGP3* is not coregulated with genes involved in glucosinolate
502 synthesis pathways or the sulfur assimilation pathway. This is possibly because *GGP3*
503 is a redundant gene of *GGP1* and its function is limited, considering the expression of
504 *GGP3* was slightly but significantly promoted in the *ggp1* knockout mutant under
505 sulfur-deficient conditions (Fig. **5b**), where there was high demand for Cys. *GGP1* is
506 presumed to contribute to GSH degradation more than *GGP3* because *GGP1* tends to be
507 expressed more actively than *GGP3* (Fig. **2**), corresponding with the fact that 335 ESTs
508 are associated with *GGP1*, whereas *GGP3* has only 66 ESTs according to TAIR.

509

510 **Expression of *GGP1* is active in mature organs and that of *GGCT2;2* is active in**
 511 **young organs**

512 The results of the complementation assay of yeast and *in vitro* activity assay
 513 showed that GGP1, GGP3, GGCT2;1, GGCT 2;2, and GGCT 2;3 solely degraded GSH
 514 without forming heterodimers (Fig. 1; Table 1). In addition, GGCT2;1 (Paulose *et al.*,
 515 2013) are localized in the cytosol and GGCT 2;2 and GGCT 2;3 are also predicted to be
 516 cytosolic in the TAIR database, so it is interesting to consider their functional
 517 differences.

518 The expression patterns of *GGPs* and *GGCTs* differed depending on the organ
 519 type (Fig. 2). The expression of *GGP1* and *GGP3* was more active in mature organs
 520 than in young ones, like that in mature leaves than young leaves (Fig. 2a), and in rosette
 521 leaves than cauline leaves (Fig. 2c). More specifically, GGP1 is localized in the vascular
 522 tissues of leaves (Geu-Flores *et al.*, 2011). Therefore, GSH degradation by GGP1 is
 523 considered functional in the distribution of constituent amino acids in the vascular tissue
 524 of leaves, contributing to the subsequent synthesis of proteins and other metabolites. For
 525 instance, Cys is the precursor molecule of numerous sulfur-containing metabolites such
 526 as methionine, vitamins, cofactors, and Fe-S clusters. Methionine can also be processed
 527 into *S*-adenosyl-L-methionine or ethylene (Romero *et al.*, 2014). Therefore, the
 528 contribution of GGP1 to the synthesis of proteins and their metabolites should be further
 529 studied to fully understand the physiological significance of GGP1.

530 In contrast, the expression of *GGCT2;2* was more active in young organs than
 531 in old ones, such as in young leaves than mature leaves (Fig. 2a), in cauline leaves than
 532 rosette leaves (Fig. 2c), and in lower roots than middle or upper roots (Fig. 2d). These
 533 data mostly coincide with public microarray data visualized by Genevestigator

(<https://www.genevestigator.com/>) (Fig. S6). GGCT2;2 has a high affinity and high activity compared with other GGP s and GGCTs. The K_m of GGCT2;2 is 1.7 mM (Kumar *et al.*, 2015), which is lower than that of GGP1 (5.0 mM, Table 1), GGP3 (3.7 mM, Table 1), and GGCT2;3 (6.7 mM, Table 1; 4.9 mM, Kumar *et al.*, 2015). The k_{cat} of GGCT2;2 is *c.* 5.7-fold higher than that of GGCT2;3 (Kumar *et al.*, 2015), whereas the k_{cat} of GGP1 was almost the same as that of GGCT2;3, and the k_{cat} of GGP3 was 3.8-fold higher than GGCT2;3 (Table 1). Therefore, GSH in young organs is presumed to be actively degraded by GGCT2;2. The physiological function of GGCT2;2 is possibly to provide developing organs with constituent amino acids to support plant growth. The involvement of GGCT2;2 in plant growth can be inferred from the fact that *GGCT2;1*—a gene explicitly expressed under sulfur deficiency—is related to root architecture under sulfur deficiency (Joshi *et al.*, 2019). It is interesting to investigate whether GGCT2;2 is related to root architecture under normal conditions.

The model of cytosolic GSH degradation in plants under normal and sulfur-deficient conditions

Our results of *in vitro* activity assays of GGP1, GGP3, and GGCT2;3 (Fig. S3; Table 1) together with the previous reports on GGCT2;1 (Paulose *et al.*, 2013), GGCT2;2, and GGCT2;3 (Kumar *et al.*, 2015) show that all of these enzymes can be functional under physiological GSH concentrations in the cytosol of plants. Therefore, we developed a model of cytosolic GSH degradation (Fig. 6), mainly based on transcript levels.

The GGP1 transcript was the most abundant in mature organs among GGP s and GGCTs (Fig. 2, 5), which is consistent with the high number of GGP1 ESTs in the

TAIR database and the fact that all clones that complemented the *dug2Δ* and *dug3Δ* yeast mutants corresponded to *GGP1*. In our mutant analysis, *ggp1* knockout mutants showed GSH accumulation under normal and sulfur- and nitrogen-deficient conditions (Fig. 4, S5). Therefore, we assumed that GGP1 generally contributes to GSH degradation in mature organs (Fig. 6). Under sulfur deficiency, the transcript levels of *GGCT2;1* and *GGCT2;2* increased by 31-fold and 2.3-fold, respectively (Fig. 3c,d), so their contribution, especially that of *GGCT2;1*, is presumed to increase (Fig. 6). Because GSH concentration is low under sulfur deficiency, *GGCT2;1* and *GGCT2;2*, which have a higher affinity for GSH than *GGP1*, *GGP3*, and *GGCT2;3* (Table 1; Paulose *et al.*, 2013; Kumar *et al.*, 2015), may be more efficient under these conditions. In addition, *GGCT2;2* transcripts were abundant under normal conditions in developing organs such as young leaves and root tips (Fig. 2, S7); therefore, the contribution of *GGCT2;2* is considered larger in these organs.

The degradation pathway through GGPs is more efficient than GGCTs because the former directly produces Glu without ATP (Geu-Flores *et al.*, 2011), whereas the latter requires ATP to convert 5-oxoproline to Glu (Ohkama-Ohtsu *et al.*, 2008). Thus, it is conceivable that plants fundamentally degrade GSH with GGPs and utilize GGCTs only when the demand for GSH degradation is high; thus, high-affinity or high-activity enzymes are needed in conditions where organs are developing or under sulfur deficiency. Notably, our findings suggest that plants utilize two types of GSH degradation pathways to meet the demand for constituent amino acids with less energy.

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585

586 Author contributions

587 T.I., T.K., K.N., M.U. and N. O-O. designed the experiments and analyzed the data; T.I.,
588 T.K., K.N., M.U., S.M., S.A. and K.K. performed experiments; A. M-N., R. S., M.Y.H.,
589 T.Y. and N.O-O. supervised the experiments; T.I., A. M-N., R. S., M.Y.H. and N.O-O.
590 contributed to the writing of the manuscript.

591

592 Data availability

593 The data that supports the findings of this study are available in the supplementary
594 material of this article.

595

596 **Table 1** Kinetic parameters of GGP1, GGP3, and GGCT2;3.

Enzyme	k_{cat} (min ⁻¹)	K_m (mM)	k_{cat}/K_m (min ⁻¹ mM ⁻¹)
GGP1	19.9	5.0	4.0
GGP3	70.3	3.7	19.0
GGCT2;3	18.5	6.7	2.8

597

598

599 **FIGURE LEGENDS**

600 **Fig. 1** Functional complementation assay of *Arabidopsis* γ -glutamyl peptidase 1
 601 (*GGP1*), γ -glutamyl cyclotransferase 2;1 (*GGCT2;1*), *GGCT2;2* and *GGCT2;3* in yeast
 602 mutants. The *Saccharomyces cerevisiae* *dug2Δ* (a) or *dug3Δ* (b) mutants (DUG:
 603 Defective in Utilization of Glutathione) were transformed with plasmids carrying the
 604 *Arabidopsis* genes *GGP1*, *GGCT2;1*, *GGCT2;2*, or *GGCT2;3* driven by the TEF
 605 promoter in the p416TEF vector. Yeast transformants were grown in an SD medium
 606 supplemented with glutathione (GSH). The vector p416TEF was used as a control, and
 607 the growth of untransformed *dug2Δ* (a) or *dug3Δ* (b) is also shown.

608
 609 **Fig. 2** Normalized copy numbers of *GGP1*, *GGP3*, *GGCT2;1*, *GGCT2;2*, and *GGCT2;3*
 610 transcripts in various organs of wild-type plants. Plants were grown in hydroponic
 611 culture. Transcript copy numbers of *GGP1*, *GGP3*, *GGCT2;1*, *GGCT2;2*, and *GGCT2;3*
 612 were analyzed by quantitative RT-PCR using the absolute quantification method and
 613 normalized by those of β -tubulin. (a) Comparison of young and old leaves from the
 614 vegetative-stage samples. (b-d) Results of the reproductive stage samples. (b) Results of
 615 flowers/buds, siliques, and stems. (c) Comparison of the rosette and cauline leaves of
 616 reproductive stage samples. (d) Comparison of the upper, middle, and lower roots of the
 617 reproductive stage samples. The values and error bars represent the mean and standard
 618 deviation of four or five biological replicates. In (a) and (c), asterisks indicate
 619 significant differences (Student's *t*-test, $P < 0.05$). In (d), different letters indicate
 620 significant differences (Tukey's test, $P < 0.05$).

621

Fig. 3 Changes in expression of the genes *GGP1*, *GGP3*, *GGCT2;1*, *GGCT2;2*, *GGCT2;3* under sulfur and nitrogen deficiency in wild-type plants. Plants were grown in flask culture. Expression levels under various nutritional conditions were measured by quantitative RT-PCR using the absolute quantification method, normalized by those of *β-tubulin*, and shown as control at 1. The values and error bars represent the mean and standard deviation of four to six biological replicates. Asterisks indicate significant differences from the control (Dunnett's test, $*P < 0.05$, $**P < 0.01$). The expression levels of (a) *GGP1*, (b) *GGP3*, (c) *GGCT2;1*, (d) *GGCT2;2*, and (e) *GGCT2;3* are shown.

Fig. 4 GSH concentrations in wild-type (WT) plants and *ggp1-1* and *ggct2;1-2* mutant plants grown for 14 d in liquid culture. The GSH concentrations in the seedlings are presented. The values and error bars represent the mean and standard deviation of four biological replicates. Asterisks indicate significant differences from the wild-type (Dunnett's test, $*P < 0.05$, $**P < 0.01$).

Fig. 5 Normalized copy numbers of *GGP* and *GGCT* transcripts in wild-type (WT) plants and *ggp1-1* and *ggct2;1-2* mutant plants under control condition and sulfur and nitrogen deficiency. The plants were grown in liquid culture for 14 d. The entire body of the 14 d-old plants was sampled. Transcript copy numbers of *GGP1*, *GGP3*, *GGCT2;1*, *GGCT2;2*, and *GGCT2;3* were analyzed by quantitative RT-PCR using the absolute quantification method and normalized by those of *β-tubulin*. The values and error bars represent the mean and standard deviation of four to six biological replicates. Asterisks indicate significant differences compared to WT (Dunnett's test, $*P < 0.05$, $**P < 0.01$).

646 Relative copy numbers of (a) *GGP1*, (b) *GGP3*, (c) *GGCT2;1*, (d) *GGCT2;2*, and (e)
647 *GGCT2;3* are shown.

648

649 **Fig. 6.** Model of cytosolic degradation of GSH in plants. GGP1 is considered to be the
650 main enzyme which degrades GSH in mature organs, and GGP3 is presumed to support
651 it. GGCT2;1 accelerates the degradation under sulfur deficiency. The expression of
652 GGCT2;3 is constitutive.

653

654

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(a)

(b)

Fig. 1 Functional complementation assay of *Arabidopsis* γ -glutamyl peptidase 1 (*GGP1*), γ -glutamyl cyclotransferase 2;1 (*GGCT2;1*), *GGCT2;2* and *GGCT2;3* in yeast mutants. The *Saccharomyces cerevisiae* *dug2Δ* (a) or *dug3Δ* (b) mutants (DUG: Defective in Utilization of Glutathione) were transformed with plasmids carrying the *Arabidopsis* genes *GGP1*, *GGCT2;1*, *GGCT2;2*, or *GGCT2;3* driven by the TEF promoter in the p416TEF vector. Yeast transformants were grown in an SD medium supplemented with glutathione (GSH). The vector p416TEF was used as a control, and the growth of untransformed *dug2Δ* (a) or *dug3Δ* (b) is also shown.

Fig. 2 Normalized copy numbers of *GGP1*, *GGP3*, *GGCT2;1*, *GGCT2;2*, and *GGCT2;3* transcripts in various organs of wild-type plants. Plants were grown in hydroponic culture. Transcript copy numbers of *GGP1*, *GGP3*, *GGCT2;1*, *GGCT2;2*, and *GGCT2;3* were analyzed by quantitative RT-PCR using the absolute quantification method and normalized by those of β -tubulin. (a) Comparison of young and old leaves from the vegetative-stage samples. (b-d) Results of the reproductive stage samples. (b) Results of flowers/buds, siliques, and stems. (c) Comparison of the rosette and cauline leaves of reproductive stage samples. (d) Comparison of the upper, middle, and lower roots of the reproductive stage samples. The values and error bars represent the mean and standard deviation of four or five biological replicates. In (a) and (c), asterisks indicate significant differences (Student's *t*-test, $P < 0.05$). In (d), different letters indicate significant differences (Tukey's test, $P < 0.05$).

Fig. 3 Changes in expression of the genes *GGP1*, *GGP3*, *GGCT2;1*, *GGCT2;2*, *GGCT2;3* under sulfur and nitrogen deficiency in wild-type plants. Plants were grown in flask culture. Expression levels under various nutritional conditions were measured by quantitative RT-PCR using the absolute quantification method, normalized by those of β -tubulin, and shown as control at 1. The values and error bars represent the mean and standard deviation of four to six biological replicates. Asterisks indicate significant differences from the control (Dunnett's test, * $P < 0.05$, ** $P < 0.01$). The expression levels of (a) *GGP1*, (b) *GGP3*, (c) *GGCT2;1*, (d) *GGCT2;2*, and (e) *GGCT2;3* are shown.

Fig. 4 GSH concentrations in wild-type (WT) plants and *ggp1-1* and *ggct2;1-2* mutant plants grown for 14 d in liquid culture. The GSH concentrations in the seedlings are presented. The values and error bars represent the mean and standard deviation of four biological replicates. Asterisks indicate significant differences from the wild-type (Dunnett's test, * $P < 0.05$, ** $P < 0.01$).

Fig. 5 Normalized copy numbers of *GGP* and *GGCT* transcripts in wild-type (WT) plants and *ggp1-1* and *ggct2;1-2* mutant plants under control condition and sulfur and nitrogen deficiency. The plants were grown in liquid culture for 14 d. The entire body of the 14 d-old plants was sampled. Transcript copy numbers of *GGP1*, *GGP3*, *GGCT2;1*, *GGCT2;2*, and *GGCT2;3* were analyzed by quantitative RT-PCR using the absolute quantification method and normalized by those of β -tubulin. The values and error bars represent the mean and standard deviation of four to six biological replicates. Asterisks indicate significant differences compared to WT (Dunnett's test, * $P < 0.05$, ** $P < 0.01$). Relative copy numbers of (a) *GGP1*, (b) *GGP3*, (c) *GGCT2;1*, (d) *GGCT2;2*, and (e) *GGCT2;3* are shown.

GSH degradation model in mature organs

Fig. 6 Models of cytosolic GSH degradation in plants. GGP1 is considered to be the main enzyme which degrades GSH in mature organs, and GGP3 is presumed to support it. GGCT2;1 accelerates the degradation under sulfur deficiency. The expression of GGCT2;3 is constitutive.