Short Title: Photorespiratory mechanism in conifer leaves

Conifer leaves have a peroxisomal oxidative decarboxylation path in the photorespiratory pathway

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One sentence summary: Conifer peroxisome is not a major localization of catalase and yields a unique oxidative decarboxylation path in the photorespiratory pathway.

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35 Abstract

36 The photorespiratory pathway consists of enzymes operating in chloroplasts, mitochondria, and
 37 peroxisomes. Conifer leaves lack one of them, chloroplastic Gln synthetase, which questioned
 38 the current belief that the photorespiratory mechanism is identical between angiosperm C₃
 39 species and conifers. A photorespiratory-metabolite analysis of the leaves of 13 conifer and 14
 40 angiosperm tree species revealed significant differences in the mean metabolite concentrations
 41 between the two taxonomic groups: the glycerate content on chlorophyll basis in conifer leaves
 42 was <1/10 that detected in angiosperm leaves, whereas the glycolate content was 1.6 times
 43 higher in conifer leaves. Glycerate is produced from Ser through an intermediate,
 44 hydroxypyruvate. To investigate the lower glycerate levels observed in conifer leaves, we
 45 performed experiments of ¹³C-labeled Ser feeding to the detached shoots of a conifer
 46 (*Cryptomeria japonica*) via the transpiration stream, and compared the labeling patterns of
 47 photorespiratory metabolites with those of an angiosperm (*Populus nigra*). Glycerate was most
 48 labeled in *P. nigra*, whereas glycolate was more labeled than glycerate in *C. japonica*. The
 49 photorespiration pathway involves H₂O₂-scavenging and H₂O₂-generating enzymes, catalase
 50 (CAT) and glycolate oxidase (GLO), respectively, which are the peroxisomal targeting enzymes
 51 in angiosperms. In contrast, database analyses of the peroxisomal targeting signal motifs and
 52 analyses of the peroxisomal fractions isolated from *C. japonica* leaves indicated that the conifer
 53 peroxisomes were not a major localization of CAT. These results suggest that the conifer
 54 photorespiration pathway has a bypass from Ser to glycolate via the decarboxylation of
 55 hydroxypyruvate, because of an imbalance between CAT and GLO activities in peroxisomes.

56 **Introduction**

57 Photorespiration starts with the oxygenation of ribulose-1,5-bisphosphate (RuBP) by the
58 CO₂-fixation enzyme Rubisco. The oxygenation reaction with one molecule of RuBP produces
59 one molecule each of 2-phosphoglycolate (2-PG) and the Calvin–Benson cycle intermediate
60 3-phosphoglycerate (3-PGA). 2-PG is a toxic product in plants and is converted to 3-PGA
61 through the photorespiratory pathway, requiring not only CO₂ emission, but also energy (ATP
62 and reductants such as NADH and reduced ferredoxin) consumption. The pathway consists of at
63 least 15 enzymes, including chloroplastic, mitochondrial, and peroxisomal enzymes, as well as
64 transport proteins to transfer the intermediates between the cell compartments (Reumann and
65 Weber, 2006; Reumann et al., 2007). Studies using *Arabidopsis* and barley mutants contributed
66 to the clarification of the enzymes that participate in the photorespiratory pathway (Ogren,
67 1984; Somerville, 2001).

68 The glutamine synthetase (GS) located in chloroplasts, GS2, is an essential enzyme of
69 the photorespiratory pathway (Wallsgrave et al., 1987). GS2 assimilates the ammonia (NH₃) that
70 is produced during the Gly-to-Ser conversion mediated by glycine decarboxylase complex
71 (GDC) reactions in mitochondria. Conversely, GS2 is absent from the leaves of conifers, and it
72 is considered that one of the cytosolic types of GS could be a substitute (Suárez et al., 2002;
73 Cánovas et al., 2007; Miyazawa et al., 2018). The leaves of conifers such as *Cryptomeria*
74 *japonica* and *Pinus densiflora* exhibited higher NH₃ emission potentials than did angiosperm
75 leaves, indicating a lowered NH₃ assimilation rate during photorespiration in conifers
76 (Miyazawa et al., 2018). These findings question our current belief that the photorespiratory
77 mechanism of conifer leaves is identical to that of angiosperm species.

78 The rate of photorespiratory CO₂ release concomitantly with the production of NH₃ by
79 the GDC reactions affects the net CO₂ assimilation rates (*A*) of the leaves. The photosynthesis
80 model (Farquhar–von Caemmerer–Berry model [FvCB model]) describes *A* via the following
81 equation (Farquhar et al., 1980):

82

$$A = V_c - \alpha \cdot V_o - R_{day}, \quad (1)$$

84

85 where V_c and V_o denote the carboxylation and oxygenation rates of Rubisco, respectively; And α
86 and R_{day} are the rate of photorespiratory CO_2 release per unit V_o and the mitochondrial
87 respiration rate in the light, respectively. By introducing the chloroplastic CO_2 compensation
88 points (I^* ; Farquhar et al. 1980), A is then given by:

89

$$A = \left(1 - \frac{I^*}{C}\right) V_c - R_{day}, \quad (2)$$

91 where

$$I^* = \frac{\alpha \cdot O}{S_{c/o}}, \quad (3)$$

93

94 where C and O are the CO_2 and O_2 concentrations in chloroplasts, respectively; and $S_{c/o}$ denotes
95 the Rubisco specificity factor. The α value is frequently assumed to be 0.5 because one molecule
96 of CO_2 during the GDC reaction is released per every two molecules of 2-PG (Farquhar et al.,
97 1980).

98 The I^* value is crucial because it determines the rate of photorespiratory CO_2 release
99 at a given C . When α is set to 0.5 and O is constant, the I^* values are calculated from *in vitro*
100 determinations of the $S_{c/o}$ of Rubisco purified from leaves, which have been intensively reported
101 for crop species (Hermida-Carrera et al., 2016). I^* values in tree species have rarely been
102 reported, perhaps because of the difficulties in determining $S_{c/o}$ in vitro. Conversely, without
103 direct measurements of $S_{c/o}$ in vitro and any assumptions of α , our previous study demonstrated
104 that I^* could be estimated via an in vivo O_2 response analysis of leaf CO_2 compensation points
105 (I , the CO_2 concentrations in the air surrounding the leaves when A equals zero), and that the
106 estimated I^* values of conifers such as *C. japonica* and *Metasequoia glyptostroboides* using

107 this in vivo method did not significantly differ from those of herbaceous species, such as
 108 tobacco and French bean (Miyazawa et al., 2020). However, a similar F^* does not ensure that
 109 conifers have the same photorespiratory mechanism as angiosperms, because the efflux of CO₂
 110 escaping from inside the leaves via the photorespiratory pathway can be less than 0.5 or more in
 111 some instances (Cousins et al., 2008, 2011; Busch et al., 2013, 2020).

112 To address the question of whether conifer leaves have a different pathway from the
 113 orthodox photorespiratory pathway, first, we compared the photorespiratory-metabolite
 114 concentrations in leaves between various conifer and angiosperm tree species grown in a field
 115 site. Furthermore, we conducted experiments using the saplings of an angiosperm tree (*Populus*
 116 *nigra*) and a conifer (*C. japonica*), which were both cultivated in a growth chamber—gene
 117 information for the two species is provided in our public cDNA library (Forest EST and
 118 Genome database: ForestGEN)—to unravel the physiological mechanisms that are responsible
 119 for the differences in the photorespiratory-metabolite profiles detected between conifer and
 120 angiosperm tree leaves.

121

122

123 **Results**

124 *Photorespiratory metabolites*

125 We compared the concentrations of the photorespiratory metabolites of leaves between 13
126 conifer and 14 angiosperm tree species (Figure 1; Supplemental Table S1). The concentrations
127 of all metabolites were expressed per unit leaf chlorophyll concentration. The metabolite
128 concentrations were analyzed using the leaf samples collected in two consecutive years. The
129 observed trends in the differences between conifers and angiosperm trees were unaffected by
130 their sampling years. The results presented in Figure 1 were summarized on a map of the
131 orthodox photorespiratory pathway (Figure 2).

132 We found clear differences in the concentrations of several metabolite between the two
133 taxonomic groups: the mean concentration of glycerate in conifer leaves was less than one-tenth
134 that detected in angiosperm leaves (Figure 2). In contrast, the glycolate and glyoxylate
135 concentrations in conifer leaves were 1.6 and 2.5 times higher than those observed in
136 angiosperm leaves, respectively. The concentrations of 2-oxoglutarate (2-OG), Gln, and NH_4^+
137 in the GS–GOGAT cycle also differed: the concentration of 2-OG in conifer leaves was about
138 one-tenth that of angiosperm tree leaves, whereas those of Gln and NH_4^+ in conifer leaves were
139 about twice higher than those recorded in angiosperm tree leaves. The concentrations of amino
140 acids such as Gly, Ser, and Glu did not differ significantly between the two groups. Significantly
141 lower levels of glycerate and 2-OG were also detected in conifer leaves when the metabolite
142 concentrations were expressed per unit leaf fresh weight (Supplemental Figure S1).

143

144 *¹³C-Ser labeling*

145 In the orthodox photorespiration pathway, glycerate is produced from Ser through the sequential
146 reactions of Ser-glyoxylate aminotransferase (SGAT) and hydroxypyruvate reductase (HPR)
147 (Figure 2). To examine the mechanisms underlying the lower levels of glycerate detected in
148 conifer leaves, we carried out a ¹³C-labeled Ser (¹³C-Ser) feeding experiment using detached

Figure 1

Comparison of leaf photorespiratory-metabolite concentrations on a chlorophyll basis between 14 angiosperm tree (Ang) and 13 conifer (Con) species grown in a field site. The left and right panels of each metabolite represent the samples collected in 2019 and 2020, respectively. The central box in each box plot represents the interquartile range and median; the whiskers indicate the 10th and 90th percentiles. The open circles indicate the average values. The scatter plots represent the data distributions. Mann-Whitney *U* tests were conducted for the pairwise comparisons between angiosperms and conifers: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

149 shoots of a conifer, *C. japonica*. We examined which photorespiratory metabolite was labeled
150 with ¹³C using gas chromatography-mass spectrometry (GC-MS) and compared the results with

Figure 2

Difference in the metabolite concentrations of the orthodox photorespiratory pathway between the leaves of angiosperm trees and those of conifers. The values adjacent to each metabolite species represent the ratio of the two-year average concentration of the 13 conifer species to that of the 14 angiosperm tree species (Supplemental Table S1). The measured metabolites are boxed, and those that showed statistically significant differences between the two taxonomic groups are shown in color based on the calculated ratios (blue, decrease; orange, increase). Fd(ox), oxidized ferredoxin; Fd(red), reduced ferredoxin; 2-OG, 2-oxoglutarate; OAA, oxaloacetate; OH-Pyr, hydroxypyruvate; 2-PG, 2-phosphoglycolate; 3-PGA, 3-phosphoglycerate; RuBP, ribulose-1,5-bisphosphate. The enzymes are indicated by circles (peroxisome-targeting enzymes are denoted in white letters in dark-green circles): CAT, catalase; GLO, glycolate oxidase; GDC, glycine decarboxylase complex; GGAT, glutamate-glyoxylate aminotransferase; GLYK, glycerate kinase; GOGAT, glutamate-2-OG aminotransferase; GS, glutamine synthetase; HPR, hydroxypyruvate reductase; MDH, malate dehydrogenase; PGLP, phosphoglycolate phosphatase; SGAT, serine-glyoxylate aminotransferase.

151 the labeling patterns of an angiosperm tree, *P. nigra*.

152 As inferred from the pathway, glycerate was mainly labeled with ¹³C in *P. nigra*

Figure 3

Heatmap visualization of the leaf photorespiratory metabolites labeled with a stable isotope of carbon (^{13}C). Ser (1.6 or 3.2 mM) was prepared so that its C3 position was labeled with ^{13}C (^{13}C -Ser) and was then fed to the detached shoots of an angiosperm tree (*Populus nigra*) (A) and those of a conifer (*Cryptomeria japonica*) (B) via the transpiration stream. As a control, unlabeled standard Ser (uSer) feedings were also carried out. The feedings were conducted over 20 or 50 min under illumination in an environmentally controlled growth cabinet. The row displays the metabolite species; the column displays different treatments. The brightness of the red color corresponds to the magnitude of the increases in the relative abundance of the isotopologue, which had a single ^{13}C atom for each metabolite (Student's *t*-test; $P < 0.05$ between the ^{13}C -Ser and uSer feedings); the yellow color indicates the absence of significant differences. The number of shoot samples used in each treatment per species was 3–4 (Supplemental Table S2).

153 (Figure 3; Supplemental Table S2). Conversely, the most labeled metabolite in *C. japonica* was
 154 glycolate, although glycerate was also labeled in this species: glycolate was 2–4.5 times more

155 labeled than glycerate. In both tree species, other photorespiratory metabolites, such as 2-OG,
156 Glu, and Gln, were not significantly labeled, with the exception of Gly. Thus, the metabolite
157 labeling pattern was different between *P. nigra* and *C. japonica*.

158

159 *Enzyme activities*

160 Glycerate is produced from hydroxypyruvate (OH-Pyr) by HPR (Figure 2). Therefore, we
161 examined whether a difference in the leaf HPR activities between the two taxonomic groups
162 could explain the observed difference in leaf glycerate contents. We found no significant
163 difference in the mean HPR activity between the angiosperm trees and the conifers in the leaves
164 collected in 2019 (Figure 4). Regarding the samples collected in 2020, the mean HPR activity of
165 the conifer leaves was slightly lower than that of the angiosperm leaves. Thus, the activities of
166 leaf HPR did not fully explain the lower glycerate levels detected in conifer leaves.

167 An in vitro experiment using isolated peroxisomes from the leaves of spinach beet
168 (*Beta vulgaris*) suggested that OH-Pyr is decarboxylated via a non-enzymatic attack of H₂O₂,
169 and is then converted to glycolate (Walton and Butt, 1981). Peroxisomes contain
170 H₂O₂-generating and H₂O₂-scavenging enzymes, i.e., glycolate oxidase (GLO) and catalase
171 (CAT), respectively. We predicted that conifer leaves would show a reduction in CAT activities,
172 and that H₂O₂ would remain and react with OH-Pyr, thus explaining the lower glycerate and
173 higher glycolate concentrations observed in the conifer leaves (Figure 1). Regarding the leaves
174 collected in 2019, there were no significant difference in the CAT and GLO activities between
175 angiosperms and conifers (Figure 4). In turn, in the samples collected in 2020, the mean CAT
176 activity of the conifer leaves was slightly lower than that of the angiosperm leaves, whereas the
177 activities of GLO and CAT were also decreased. Taken together, these results led us to conclude
178 that there was not a specific reduction in CAT activities in the conifer leaves.

179

Figure 4

The activities of three enzymes, i.e., hydroxypyruvate reductase (HPR), catalase (CAT), and glycolate oxidase (GLO), were determined in the leaves of 14 angiosperm tree (Ang) and those of 13 conifer (Con) species grown in a field site. The activities were expressed per unit soluble protein content. The left and right panels represent samples collected in 2019 and those collected in 2020, respectively. The central box in each box plot represents the interquartile range and median; the whiskers indicate the 10th and 90th percentiles. The open circles indicate the average values. The scatter plots represent the data distributions. Mann–Whitney *U* tests were conducted for pairwise comparisons between conifers and angiosperms: *, *P* < 0.05; **, *P* < 0.01.

180 *Gene expression and amino acid structure of conifer catalases*

181 The CAT and GLO proteins are peroxisomal targeting enzymes that physically interact with

each other, thus contributing to the efficient scavenging of GLO-generated H₂O₂ in rice leaves (Zhang et al., 2016). This finding intrigued us to reconsider the cellular localization of these enzymes in conifers, first based on amino acid sequences.

Analyses of the cDNA libraries revealed that *P. nigra* and *C. japonica* carried three and two genes that encode CAT enzymes, respectively, which we termed *PnCAT1–3* for *P. nigra* and *CjCATA* and *CjCATB* for *C. japonica*. We confirmed the expression of these *CAT* genes in planta using qRT-PCR, the results of which indicated that the overall expression of the *CAT* genes was higher in the leaves than in the roots of *P. nigra*, whereas it was higher in the roots vs. the leaves of *C. japonica*. In *P. nigra*, the expression of *PnCAT2* was predominant among these three genes in the leaves (Figure 5, A). The expression of *PnCAT1* and *PnCAT2* was higher in the leaves than it was in the roots, whereas *PnCAT3* was almost equally expressed in the two organs. In *C. japonica*, *CjCATA* was expressed at a higher level in the leaves than in the roots, whereas the opposite pattern was detected for *CjCATB* (Figure 5, A).

The 13 amino acids located at the carboxy-terminal (C-terminal) regions of CAT enzymes contain a peroxisomal targeting signal (PTS) motif, which was elucidated by in vivo subcellular targeting analyses, such as those using tobacco BY-2 cells and transgenic *Arabidopsis* plants (Mhamdi et al., 2010). The extreme C-terminal tripeptide is a sequence that is necessary for the efficient import of cottonseed CAT into BY-2 peroxisomes (designated as the P-S/T/N-M/I sequence by Mullen et al., 1997; Mhamdi et al., 2010).

We compared the deduced amino acid sequences of PnCATs and CjCATs, which exhibited overall high similarities between their CAT protein sequences, with the exception of a low similarity in the C-terminal regions (Figure 5, B). Moreover, we investigated the C-terminal sequences of CATs from 18 angiosperm species and nine coniferous species, and found that the Pro residue located at position –3 from the C terminus was well conserved among angiosperm CATs, including PnCAT1–3, with the exception of one of the maize CATs (ZmCAT3, NP_001350821.1) (Figure 5, B and C; Supplemental Table S3). In ZmCAT3, Pro was replaced

Figure 5
Differences in the gene expression and protein sequences of catalase (CAT) between angiosperms and conifers. **A**, Relative mRNA expression levels of the CAT genes in the leaves and roots of an angiosperm tree (*Populus nigra*) (left, *PnCAT1*–3) and a conifer (*Cryptomeria japonica*) (right, *CjCATA* and *CjCATB*) saplings. The open and hatched bars represent the leaves and roots, respectively. The expression levels of the CAT genes were normalized against the expression level of the actin-encoding genes. Mean $\pm$ SD, $n = 3$ biological replicates. **B**, Alignment of the amino acid sequences of the CAT proteins of *P. nigra* with those of *C. japonica*. Identical amino acid regions are indicated in gray. The region of the peroxisomal targeting signal (PTS), which has been implicated in the regulation of the import into peroxisomes, is boxed. **C**, Weblogo images of the PTS sequences of the CAT proteins for angiosperms and conifers. To generate the images, we referred to 51 genes from 18 angiosperm species, including herbs, grasses, and trees; and 13 genes from nine coniferous species, based on databases (Supplemental Table S3).

208 with Ala. An organelle fractionation study indicated that ZmCAT3 was located in the
209 mitochondrial fractions (Scandalios et al., 1980). Moreover, an analysis of the deduced amino

acid sequences retrieved from several databases showed a clear difference in this amino acid between angiosperms and conifers: Pro was replaced with either Ala or Ser in all studied conifer CAT enzymes, including CjCATA and CjCATB (Figure 5, B and C). In contrast, the amino acids from positions -4 to -13 in the PTS did not show a specific difference between the angiosperm and the conifer CAT enzymes. Based on these results, we concluded that the conifer CAT enzymes were not localized at, or were less imported into, the peroxisomes.

Unlike CAT enzymes, an extreme C-terminal tripeptide functions as a PTS in GLO enzymes (Reumann et al., 2006). Based on the conifer database analyses, the C-terminal sequence of their GLOs exhibited the major PTS motifs, either S-K-L or S-R-L (Supplemental Table S4). Both motifs were experimentally verified by in vivo subcellular targeting analyses using transgenic *Arabidopsis* plants (Hayashi et al., 1997). Similarly, other photorespiration-related enzymes, including glutamate-glyoxylate aminotransferase (GGAT), HPR, malate dehydrogenase (MDH), and SGAT—which are the peroxisomal targeting enzymes in angiosperms—possessed the experimentally verified PTS motifs in all studied conifers (Supplemental Table S5).

Peroxisome fractions isolated from Populus and Cryptomeria leaves

Our sequence analyses on the PTS motifs suggest that the conifer peroxisomes are not a major localization of CAT enzymes (Figure 5). To examine this finding experimentally, we isolated peroxisomes from *C. japonica* leaves using the Percoll/sucrose density gradient method. The GLOs of *C. japonica* and *P. nigra* had experimentally verified PTS motifs, i.e., S-R-L and P-R/K-L, respectively (Supplemental Table S4; Kaur et al. 2009), which allowed us to use GLO activity as a marker for elucidating peroxisome targeting. The measurement of glycerate kinase (GLYK) and fumarase (FUM) activities can be used to evaluate contaminations with chloroplasts and mitochondria in isolated peroxisome fractions (Usuda and Edwards, 1980). The calculated average recovery rates were 0.23% and 1.2% for *P. nigra* and *C. japonica*,

Figure 6
Characteristics of the peroxisome fractions isolated from the leaves of an angiosperm tree (*Populus nigra*, *Pn*) and from the leaves of a conifer (*Cryptomeria japonica*, *Cj*) using the Percoll/sucrose density gradient method. $C_{\text{chloro.}}$, chloroplast contamination index; $C_{\text{mito.}}$, mitochondrial contamination index; L_{CAT} , peroxisomal localization index of catalase enzymes in the isolated peroxisome fractions. Mean $\pm$ SD from three-to-four independent experiments (Table 1). The values of n.d. in Table 1 were assumed to be zero for the mean calculations.

236 respectively (Table 1), which were lower than the reported value for *Arabidopsis* (3.7%;
237 Reumann et al., 2007). The calculated contamination with mitochondria ($C_{\text{mito.}}$) and chloroplasts

238 ($C_{\text{chloro.}}$) was 16% and 7% for *C. japonica*, respectively (Figure 6). For *P. nigra*, $C_{\text{mito.}}$ was 3.7%,
 239 whereas significant $C_{\text{chloro.}}$ was not detected. As indicated by the peroxisomal localization index
 240 of CAT (L_{CAT}), more than 90% of the leaf CAT was localized in peroxisomes in *P. nigra*,
 241 whereas only 13% of the leaf CAT was localized in peroxisomes in *C. japonica* (Figure 6). The
 242 low L_{CAT} calculated for *C. japonica* was consistent with the results obtained for the PTS motif
 243 (Figure 5, C).

244

245 Chloroplastic CO_2 compensation point (Γ^*)

246 The Γ^* of *P. nigra* was unknown. Therefore, the Γ^* value of *P. nigra* leaves was estimated using
 247 the same method as that applied for *C. japonica* leaves (Miyazawa et al., 2020): Γ^* was
 248 estimated through measurements of the changes in the leaf CO_2 compensation points (Γ) in
 249 response to the O_2 partial pressure in air for a variety of leaves with different rates of Rubisco
 250 maximum carboxylation (V_{cmax}) and respiration in the light (R_{day}). The calculated Γ^* values
 251 (mean $\pm$ SE) were similar between the two species: 40.1 ± 0.9 and 41.2 ± 1.6 μbar for *P. nigra*
 252 and *C. japonica*, respectively (Supplemental Table S6; Supplemental Figure S2).

253

254

255 Discussion

256 *The photorespiratory mechanism of conifers is different from that of angiosperm C_3 species*

257 The results of our metabolite and ^{13}C -labeling analyses suggest that the photorespiratory
258 mechanism is not identical between angiosperm tree and conifer leaves (Figure 2; Figure 3).
259 Our findings from the analyses of the PTS motifs and isolated peroxisome fractions suggest that
260 the H_2O_2 -scavenging enzyme, CAT, is not abundant in conifer peroxisomes (Figure 5; Figure 6).
261 Based on these findings, we propose a novel model for conifer photorespiration: a pathway from
262 Ser to glycolate via the oxidative decarboxylation of OH-Pyr with H_2O_2 , caused by an
263 imbalance between CAT and GLO activities in peroxisomes. The present study was the first
264 report showing that this decarboxylation pathway probably has a major flux in conifer
265 photorespiration, and revealing that a diversification of photorespiratory metabolism exists
266 among tree groups.

267 Previous studies have suggested that the oxidative decarboxylation of OH-Pyr occurs
268 in transgenic tobacco plants, as well as in *Arabidopsis* mutants, which have impairments in
269 peroxisome-targeting enzymes, such as CAT, HPR, and peroxisomal MDH (Brisson et al., 1998;
270 Cousins et al., 2008, 2011). For example, transgenic tobacco with reduced CAT activities
271 exhibited higher leaf CO_2 compensation points (Γ) than the wild-type plants (Brisson et al.,
272 1998). The Γ increased concomitantly with the decrease in the leaf CAT activities, probably
273 because the excess of H_2O_2 caused by insufficient CAT activity decarboxylates ketoacids such
274 as OH-Pyr and glyoxylate in peroxisomes, thereby causing a loss of assimilated CO_2 (Brisson et
275 al., 1998). This suggests that an imbalance between CAT and GLO activities in the peroxisomes
276 caused an increase in the rate of photorespiratory CO_2 release per unit V_o (α). Conversely, the
277 results pertaining to Γ warrant careful interpretation, because Γ is affected not only by the α
278 values, but also by the $R_{\text{day}}/V_{\text{cmax}}$ ratio of the leaves, as noted in Equations 9 and 10
279 (Supplemental Methods).

280 The chloroplastic CO_2 compensation point (Γ^*) is more simply defined than Γ because

281 I^* is independent of $R_{\text{day}}/V_{\text{cmax}}$ (see Equation 3). The I^* values of *Arabidopsis* mutants lacking
 282 either peroxisomal MDH or HPR were about 1.5 times higher than those of the wild-type plants,
 283 because of the increases in α triggered by oxidative decarboxylation reactions of ketoacids in
 284 peroxisomes (Cousins et al., 2008, 2011). The present study suggests that such peroxisomal
 285 oxidative decarboxylation occurs in conifer leaves. In contrast, the I^* value of *C. japonica*
 286 leaves was similar to the values reported for the leaves of wild-type angiosperm species, such as
 287 *P. nigra* (this study), tobacco, and French bean (Miyazawa et al., 2020).

288

289 *The isolation process of conifer peroxisomes warrants improvement*

290 The significantly low level of L_{CAT} detected in *C. japonica* suggests that peroxisomes possess a
 291 lower amount of CAT compared with other subcellular fractions in conifers (Figure 6).
 292 Conversely, in the present study, we were not able to determine which subcellular fraction, i.e.,
 293 mitochondria, chloroplasts, or the cytosol, etc., possessed a high level of CAT in the conifer leaf
 294 cells.

295 The peroxisome fractions of *Arabidopsis* were isolated with higher purity than ours:
 296 the organelle contaminations detected in *Arabidopsis* ($C_{\text{chloro.}}$ of 0.12% and $C_{\text{mito.}}$ of 1.7%;
 297 Reumann et al., 2007) were lower than those recorded in our studied species, particularly in *C.*
 298 *japonica* (Figure 6). Reumann et al. (2007) applied a two-step density gradient centrifugation
 299 method for the isolation of peroxisomes from *Arabidopsis* leaves: Percoll/sucrose density
 300 gradient followed by sucrose density gradient centrifugations. In our experiments, the
 301 Percoll/sucrose density gradient alone was applied for the isolation of peroxisomes. We
 302 attempted to isolate the peroxisomal fractions using the two-step method; however, we were not
 303 able to obtain measurable amounts of the peroxisomal fractions from the leaves of the two
 304 studied species. The low, but non-negligible, CAT activity detected in the isolated peroxisome
 305 fractions of *C. japonica* could be attributed to a relatively high contamination with chloroplasts
 306 and mitochondria.

307 Recently, in the leaves of rice, Zhang et al. (2016) reported that the CAT and GLO
308 proteins associated together in peroxisomes and efficiently scavenged the H₂O₂ generated by
309 GLO. In contrast, the conifer peroxisomes are not likely to possess such physical interaction
310 between the CAT and GLO proteins, because the peroxisomes were not the major subcellular
311 localization of CAT (Figure 6). However, we cannot rule out the possibility that other enzymes,
312 such as ascorbate peroxidase, potentially scavenge H₂O₂ in peroxisomes (Kaur et al., 2009) and
313 are involved in photorespiration in conifers. To elucidate more precisely the localization and
314 functions of CAT in conifer leaf cells, we will need to improve the purity of the peroxisomes
315 isolated from conifer leaves, and to perform additional subcellular fractionation analyses.

316

317 *Metabolites in the GDC and GS–GOGAT reactions*

318 In the ¹³C-Ser feeding experiments, Gly was significantly labeled with ¹³C in the two studied
319 species (Figure 3). Because glycolate was not significantly labeled with ¹³C in *P. nigra*, the
320 significant labeling of Gly in this species might result from the synthesis of Gly from Ser via the
321 reverse reaction of GDC in mitochondria (Sarojini and Oliver, 1983). It is also noteworthy that
322 the concentrations of metabolites including Gln, 2-OG, and NH₄⁺, which are involved in the
323 GS–GOGAT cycle, were significantly different between the conifer and angiosperm tree leaves
324 (Figure 2). The higher NH₄⁺ content per unit chlorophyll concentration detected in the conifer
325 leaves was in good agreement with the previous finding that conifer leaves have a higher NH₃
326 emission potential than do angiosperm leaves (Miyazawa et al., 2018). Moreover, the
327 concentration of 2-OG in the conifer leaves was only about one-tenth that of the angiosperms,
328 whereas the Gln concentration in the conifer leaves was about two times higher (Figure 2).
329 Although we do not have a straightforward elucidation of the physiological mechanisms that are
330 responsible for such differences in the GS–GOGAT metabolites between the two taxonomic
331 groups, it might be related to the fact that conifer leaves lack GS2 enzymes (Cánovas et al.,
332 2007; Miyazawa et al., 2018).

333

334 *Inconsistency between Γ^* and ^{13}C -labeling experiment results*

335 Our metabolite profile and ^{13}C -labeling experiment results suggested that conifer leaves have an
 336 additional CO_2 release from peroxisomes and, hence, a higher stoichiometry of photorespiratory
 337 CO_2 release. Conversely, the in vivo Γ^* values of the conifer leaves were similar to those of the
 338 angiosperm leaves (Miyazawa et al., 2020). As noted in Equation 3, this inconsistency can be
 339 explained by three possibilities, assuming that the chloroplastic O_2 concentration (O) is
 340 constant: (1) a higher $S_{c/o}$, (2) a higher proportion of photorespiratory CO_2 that is re-fixed by
 341 Rubisco, and (3) a lower rate of CO_2 release by the GDC reactions per V_o , in conifer leaves.

342 The three possibilities are explained in greater detail as follows. (1) Galmés et al.
 343 (2014) indicated that the CO_2 affinity of Rubisco purified from the leaves of a conifer
 344 (*Metasequoia*) was significantly lower than that of Rubisco purified from the leaves of
 345 angiosperms. Therefore, it is unlikely that the $S_{c/o}$ of conifers is higher than that of angiosperms,
 346 although the kinetic data of conifer Rubisco is insufficient, and the in vitro $S_{c/o}$ value for conifer
 347 Rubisco has not been reported to date. (2) The rate of CO_2 release escaping from inside the
 348 leaves via the photorespiratory pathway is affected by the extent to which photorespiratory CO_2
 349 is re-fixed by Rubisco (Busch et al., 2013). The extent of this process is predicted to be larger in
 350 leaves with a thicker mesophyll cell wall that imposes a larger diffusion resistance to the CO_2
 351 efflux from inside the mesophyll cells to the intercellular air space in leaves (Tholen et al.,
 352 2012; Eckert et al., 2021). Because conifer leaves have thicker mesophyll cell walls than
 353 angiosperms, such as broad-leaved trees and C_3 annual herbs (Terashima et al., 2011;
 354 Veromann-Jürgenson et al., 2017), the high stoichiometry of photorespiratory CO_2 release in
 355 conifers can be cancelled out by a high rate of CO_2 re-fixation by Rubisco. (3) The
 356 intermediates in the photorespiratory pathway, such as Gly and Ser, can be exported from the
 357 pathway and utilized in other metabolic processes, such as amino acid and secondary product
 358 syntheses, which could result in a change in the rate of CO_2 release by the GDC reactions per V_o

(Busch et al., 2020). Further studies are needed to investigate whether there are differences in the metabolic processes linked with the photorespiratory pathways between conifers and angiosperms, and whether the rate of CO₂ release by GDC reactions per V_o differs.

Conclusions

The present study suggested that the differences in the subcellular localizations of CAT and GLO yielded a bypass from Ser to glycolate via the oxidative decarboxylation of OH-Pyr in conifer photorespiration. Together with the previous finding of an absence of GS2 in conifer leaves (Cánovas et al., 2007; Miyazawa et al., 2018), the present study supported the contention that conifer gymnosperms have a different photorespiratory mechanism compared with angiosperm C₃ species. It is known that H₂O₂ decarboxylates not only OH-Pyr, but also glyoxylate, which is then converted to formate (Walton and Butt, 1981). We plan to investigate whether formate in leaves is also labeled by ¹³C after feeding ¹³C-Ser to detached shoots of *C. japonica*. The HPR requires the reductant NADH for converting OH-Pyr to glycerate (Ogren, 1984); in contrast, the oxidative decarboxylation may not have this requirement, implying that the energy requirements for the conifer photorespiratory pathway can be different from those in this pathway in angiosperms. Notably, our first observation of the differences in photorespiratory mechanisms between conifers and angiosperm C₃ species suggests that the application of the FvCB model to conifer photosynthesis needs to be revised.

Materials and Methods

Plant materials

Analyses of the photorespiratory-metabolite profiling and enzymatic activity were performed using leaf samples collected from the current-year branches of the tree species listed in Supplemental Table S1. The tree species were cultivated in an FFPRI tree garden. Sunlit leaves were collected from three individuals per species (two individuals for *Morus australis*) between

10:00 and 14:00 on sunny days in August. The collected leaf samples were pooled for each species. The samplings were carried out in 2019 and 2020. For ^{13}C -labeled Ser feeding and peroxisome-isolation experiments, we used potted saplings of *Cryptomeria japonica* and *Populus nigra*. Those plants were grown in an air-conditioned glasshouse under a natural light environment (day/night air temperature, 25°C/20°C; day/night relative humidity, 65%/60%) or under an artificial-light environment (day/night air temperature, 25°C/20°C; relative humidity, 70%; photosynthetically active photon flux density at the plant top, 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$). For gene expression analyses, the mature leaves of current-year branches and fine roots were sampled during the daytime (10:00–11:00) from three potted individuals for each species grown in the glasshouse in June for *P. nigra* and in September for *C. japonica*. The fine roots were cut with a pair of scissors and then rinsed with distilled water. The detailed growth conditions are described in the Supplemental Methods. All leaf and root samples were frozen in liquid nitrogen immediately after collection, and stored at -80°C until measurements.

Metabolite determinations

Leaf metabolites were extracted using a chloroform and methanol solvent. The detailed procedures of extraction were as described in our previous paper (Tahara et al., 2021). Photorespiration-related organic acids other than glyoxylate were quantified using a GC-MS system (7890B/5977A; Agilent Technologies, Santa Clara, CA) equipped with a DB-5MS capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness, Agilent); whereas the amino acids were quantified using an HPLC system (Agilent 1100; Agilent) equipped with a Zorbax Eclipse Plus C18 column (4.6 mm $\times$ 150 mm, 3.5 μm particle size, Agilent).

To prepare GC-MS samples, the ketone moieties of the compounds in freeze-dried leaf extracts were methoximated using 10 μL of 40 mg mL^{-1} methoxiamine hydrochloride (FUJIFILM Wako Chemicals, Osaka, Japan) in pyridine at 30°C for 90 min; the acidic protons were subsequently derivatized with 90 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide plus

411 1% trimethylchlorosilane (Thermo Fisher Scientific, Waltham, MA) at 37°C for 30 min. Ribitol
412 (final concentration, 4 µg mL⁻¹) was used as an internal standard. The GC-MS conditions were
413 exactly the those described by Tahara et al. (2021).

414 The GC-MS *m/z* values in selected ion monitoring mode were: 205, 217, 319 for
415 ribitol; 205, 217, 292, 307 for glycerate; 147, 161, 177, 205 for glycolate; and 198, 229, 288,
416 304 for 2-OG. The GC-MS retention times (in min) were: 15.6 for ribitol; 10.7 for glycerate;
417 7.08 for glycolate; and 13.8 for 2-OG. Their amounts were determined based on their peak areas
418 using authentic standards (Cat No. 1021-58400, GL Science, Tokyo, Japan). All calculations
419 were carried out using the software (MassHunter, Quantitative Analysis Ver.7.01 SP1; Agilent).
420 Glyoxylate was not within the measurable range of the GC-MS retention time. Therefore,
421 glyoxylate concentrations were spectrophotometrically determined (UV-1900; Shimadzu, Kyoto,
422 Japan) according to the method described by Häusler et al. (1996) with a slight modification.
423 We used a 0.1 M HCl solution containing 1% (w/v) phenylhydrazine (instead of the 0.1%
424 described in Häusler et al.).

425 The amino acid concentrations were determined by the HPLC method of pre-column
426 derivatization using an *o*-phthalaldehyde and 9-fluorenylmethyl chloroformate reagent. Further
427 details are described in the manufacturer manual
428 (<https://www.agilent.com/cs/library/applications/5990-4547EN.pdf>, Agilent). Norvaline (final
429 concentration, 8.4 µg mL⁻¹) was used as an internal standard. Leaf chlorophyll was extracted
430 with 80% acetone and spectrophotometrically quantified (Porra et al., 1989).

431

432 *Ammonium (NH₄⁺)*

433 Leaf NH₄⁺ concentrations were fluorometrically determined using the *o*-phthalaldehyde (OPA)
434 method in a neutral pH condition using a microplate reader (Varioscan LUX; Thermo Fisher
435 Scientific), which was based on the methods described by Husted et al. (2000). A frozen leaf
436 sample (20–40 mg of fresh weight per sample) was ground with 500 µL of 20 mM formate

437 using a chilled mortar and pestle. Aliquots of leaf extracts (20 μ L per sample) after
438 centrifugation at $16000 \times g$ (5 min at 4°C) were mixed with 180 μ L of OPA solution in a
439 96-well plate placed on crushed ice. Subsequently, the plates were gently shaken at 60°C for 3
440 min (Microincubator M-36; TAITEC Co. Saitama, Japan) to proceed the reaction of NH_4^+ in
441 extracts with OPA, and the fluorescence intensities at 470 nm (excited at 410 nm) were measured.
442 The OPA solution contained 100 mM phosphate buffer (pH 7), 29 mM OPA, and 10 mM
443 2-mercaptoethanol. NH_4NO_3 was used as a standard.

444

445 *Leaf HPR, CAT, and GLO activities*

446 For HPR activity measurements, frozen leaf samples were ground using a chilled mortar and
447 pestle and homogenized in an extraction buffer containing 100 mM HEPES-KOH (pH 7.8), 1
448 mM EDTA, 10% (v/v) glycerol, 10 mM MgSO_4 , 5 mM dithiothreitol (DTT), 0.01 mM leupeptin,
449 and 1% (v/v) Nonidet P-40. The supernatants of the extracts obtained after centrifugation were
450 used for measurements. The HPR activity was determined based on the decrease in absorbance
451 at 340 nm in an assay solution containing 100 mM HEPES-KOH (pH 6.8), 0.1 mM NADH, and
452 1 mM lithium hydroxypyruvate (Husic and Tolbert, 1987) at a controlled temperature of 30°C.
453 For CAT and GLO activity measurements, frozen leaf samples were ground using a chilled
454 mortar and pestle and homogenized in an extraction buffer containing 50 mM phosphate buffer
455 (pH 7.0), 1 mM EDTA, 10% (v/v) glycerol, 0.01 mM leupeptin, and 1% (v/v) Nonidet P-40.
456 The supernatants of the extract obtained after centrifugation were used for measurements. CAT
457 activity was determined using an O_2 electrode (Oxygraph+; Hansatech Instruments Ltd.,
458 Norfolk, UK) (del Río et al., 1977). The final concentration of H_2O_2 used as a substrate was ca.
459 180 mM. GLO activity was spectrophotometrically determined based on the method described
460 by Yamaguchi and Nishimura (2000). The measurement temperatures were controlled at 30°C.
461 Further details are described in the Supplemental Methods. The soluble protein content was
462 spectrophotometrically determined using bovine serum albumin as a standard (Bradford Protein

463 Assay Kit; Bio-Rad, Hercules, CA).

464

465 *¹³C-labeled Ser feeding*

466 Before plants were illuminated, the shoots were enclosed with aluminum foil to keep them
467 under dark conditions until the onset of the treatments. The shoots were detached from the
468 plants using a razor blade and were immediately immersed in distilled water in 50-mL tubes
469 (one shoot per tube). The shoots kept in the tubes were placed under a light intensity of 400
470 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ in an air-conditioned growth chamber (25°C and 70% relative humidity) at an
471 ambient CO₂ concentration of ca. 400 $\mu\text{mol mol}^{-1}$ for 30 min. Subsequently, labeled L-Ser, the
472 C3-position of which is mainly labeled with a stable isotope of carbon (¹³C-Ser), was added to
473 the tubes (final concentration, 1.6 or 3.2 mM). The distribution of the isotopologue abundance
474 of an unlabeled Ser standard (uSer) was similar to that detected in the leaves sampled before
475 ¹³C-Ser addition (Supplemental Figure S3). Therefore, uSer at the same concentrations as those
476 of the corresponding ¹³C-Ser treatments was used as controls. ¹³C-Ser (Cat. No. 604720) and
477 uSer (Cat. No. 54763) were purchased from MilliporeSigma (Burlington, MA). The shoots were
478 sampled 20 or 50 min after Ser additions.

479

480 *Relative isotopologue abundance*

481 The relative isotopologue abundance (m_i) of each metabolite into which $i^{13}\text{C}$ atoms were
482 incorporated was calculated using the following equation (Hasunuma et al., 2010):

483

$$484 \quad m_i = \frac{M_i}{\sum_{j=0}^n M_j}, \quad (4)$$

485

486 where M_i represents the abundance of the isotopologues into which $i^{13}\text{C}$ atoms were
487 incorporated and n is the number of C atoms that each metabolite possessed. According to Berry
488 et al. (1978) and Huege et al. (2007), M_i at the selected GC-MS m/z values was measured in the

single-ion monitoring mode (Supplemental Table S7). The major isotopologue of ^{13}C -Ser possessed a single ^{13}C atom ($m_1 = 77\%$; Supplemental Figure S3). Therefore, a ^{13}C enrichment of each photorespiratory metabolite was evaluated as a percent increase in m_1 for the leaves that were fed with ^{13}C -Ser from that recorded for the leaves that were fed with uSer.

Peroxisome isolation

Leaf peroxisomes were isolated using the Percoll/sucrose density gradient method (Reumann and Lisik, 2017), with a slight modification. Our extraction buffer contained no DTT because severe inhibitions of CAT activities by DTT have been reported (Chandlee et al., 1983). The detached leaves enclosed in a plastic bag were kept on crushed ice in the dark (2 h), for loosening the physical interactions among organelles. The leaves were ground using a mortar and pestle on ice with an extraction buffer containing 170 mM Tricine-KOH (pH 7.5), 1.0 M sucrose, 2 mM EDTA, 1% (w/v) BSA, 10 mM KCl, 1 mM MgCl_2 , and 0.5% (w/v) polyvinylpyrrolidone-40 supplemented with a protease inhibitor cocktail (cOmplete, EDTA-free; Roche, Basel, Switzerland). After the removal of cellular debris, chloroplasts, and nuclei from the extracted solution via filtration using a layer of Miracloth (Millipore, MA) and mild centrifugation ($5,000 \times g$ for 1 min), 15 mL of the supernatant was loaded onto a Percoll/sucrose density gradient prepared in TE buffer (20 mM Tricine-KOH (pH 7.5) and 1 mM EDTA) supplemented with 0.75 M sucrose and 0.2% (w/v) BSA, and underlaid by 36% (w/v) sucrose in TE buffer (top to bottom: 2.4 mL of 15% Percoll, 8.8 mL of 38% Percoll, 1.6 mL of a mixture of 38% Percoll and 36% [w/v] sucrose at a ratio of 2:1 and 1:2, and 2.4 mL of 36% [w/v] sucrose in TE buffer). A sediment fraction corresponding to crude peroxisomal fractions (LP-P1 fraction; Reumann and Lisik, 2017) was obtained by stepwise centrifugation ($13,000 \times g$ for 12 min, followed by an increase to $27,000 \times g$ for an additional 20 min at 4°C) on an ultracentrifuge (Himac SCP85H2; Hitachi, Tokyo, Japan). The isolated peroxisome fractions were stored in a freezer at -80°C until enzyme activity measurements. The CAT and

GLO activities were measured as described above. The activities of GLYK and FUM were measured and used as markers for identifying chloroplast and mitochondria contaminations, respectively, at a temperature of 30°C (Kleczkowski and Randall, 1986; Gibon et al., 2004). The contamination of leaf peroxisomes by chloroplasts and mitochondria was calculated (Reumann et al., 2007) based on the relative activities of the corresponding marker enzymes (i.e., Act_{GLYK} and Act_{FUM}) that were recovered in the leaf peroxisome (LP) fraction compared with the crude extract (CE). The activity of GLO (Act_{GLO}) was used as a peroxisome marker. For example, the contamination with chloroplasts ($C_{chloro.}$) was calculated as:

$$Act_{GLYK}(\% \text{ of CE}) = \frac{Act_{GLYK}(LP) \cdot 100}{Act_{GLYK}(CE)}, \quad (5)$$

$$C_{chloro.} = \frac{100 \cdot Act_{GLYK}(\% \text{ of CE})}{Act_{GLO}(\% \text{ of CE})}. \quad (6)$$

Using the same analogy of the contamination calculations, the peroxisome localization of CAT (L_{CAT}) was calculated as:

$$Act_{CAT}(\% \text{ of CE}) = \frac{Act_{CAT}(LP) \cdot 100}{Act_{CAT}(CE)}, \quad (7)$$

$$L_{CAT} = \frac{100 \cdot Act_{CAT}(\% \text{ of CE})}{Act_{GLO}(\% \text{ of CE})}. \quad (8)$$

Estimation of Γ^* for *Populus nigra*

We estimated the Γ^* of *P. nigra* from the analyses of the O_2 responses of the leaf CO_2 compensation points (Γ) according to the method described by Miyazawa et al. (2020), and compared it with the reported Γ^* value of *C. japonica*. The initial slopes of the relationships between the leaf net CO_2 assimilation rates (A) and the intercellular CO_2 concentrations (C_i) were obtained at different air- O_2 concentrations using a portable CO_2 gas exchange analyzer (LI-6400; Li-Cor, Lincoln, NE). The O_2 concentrations ranged from 10% to 30% (10%, 15%,

20%, 25% and 30%). The $A-C_i$ initial slope was fitted by linear regression. The value at the location on the x -axis where the linear regression lines crossed was taken as the estimate of T . Leaf temperature was controlled at 25°C. For additional details on the measurement procedures and conditions, refer to the Supplemental Methods.

Sequencing

The catalase-encoding genes of *P. nigra* and *C. japonica* were searched against cDNA libraries (ForestGEN; <http://forestgen.ffpri.affrc.go.jp/en/index.html>) using an *Arabidopsis* catalase sequence (GenBank No.; NP_564121.1) as a query on the TBLASN program. The selected cDNA clones were sequenced on a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). The deduced amino acid sequences were aligned using the MUSCLE program (GENETYX ver. 15.0.0; Genetyx, Tokyo, Japan). The sequence logos of the peroxisomal targeting regions were generated using Weblogo (<https://weblogo.berkeley.edu/logo.cgi>).

Quantitative real-time RT-PCR (qRT-PCR)

Frozen leaf samples were ground using a chilled mortar and pestle. Total RNA was isolated according to the method described by Izuno et al. (2020), and subsequently used for the synthesis of the first-strand cDNA with iScript Reverse Transcription Supermix (Bio-Rad) after checking the RNA quality (2100 Bioanalyzer; Agilent). The quantitative real-time PCR assay was performed using the SsoAdvanced SYBR Green Supermix (Bio-Rad) with specific primers (Supplemental Table S8). The expression levels of the *CAT* genes were normalized to the expression level of the respective actin-encoding genes (*PnACT* or *CjACT*).

Statistical analysis

Mann–Whiney U tests and Student’s t -tests were performed using the Stata 16.1 software (StataCorp, College Station, TX). The box plots were generated using a graphing computer

566 software (Origin 2021b; Origin Lab., Northampton, MA).

567

568   **Accession Numbers**

569   Genes referenced in this article are available at GenBank with the accession numbers:
 570   LC627279 (*PnCAT1*), LC627280 (*PnCAT2*), LC627281 (*PnCAT3*), LC627277 (*CjCATA*),
 571   LC627278 (*CjCATB*).

572

573   **Supplemental Data**

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

575   Supplemental Methods.

576   Supplemental Figure S1. Metabolite concentrations on a leaf fresh weight basis.

577   Supplemental Figure S2. Relationships between d and $R_{\text{day}}/V_{\text{cmax}}$.

578   Supplemental Figure S3. Isotopologue abundance of Ser.

579   Supplemental Table S1. Photorespiratory-metabolite concentrations.

580   Supplemental Table S2. Isotopologue abundance of the ^{13}C -Ser feedings.

581   Supplemental Table S3. PTS motifs of catalase (CAT).

582   Supplemental Table S4. PTS motifs of glycolate oxidase (GLO).

583   Supplemental Table S5. PTS motifs of photorespiratory enzymes other than CAT and GLO.

584   Supplemental Table S6. Gas exchange parameters of *P. nigra* leaves.

585   Supplemental Table S7. m/z values for the ^{13}C -Ser feedings.

586   Supplemental Table S8. List of the primers used in the CAT expression analyses.

587

589 **Figure Legends**

590 **Figure 1**

591 Comparison of leaf photorespiratory-metabolite concentrations on a chlorophyll basis between
592 14 angiosperm tree (Ang) and 13 conifer (Con) species grown in a field site. The left and right
593 panels of each metabolite represent the samples collected in 2019 and 2020, respectively. The
594 central box in each box plot represents the interquartile range and median; the whiskers indicate
595 the 10th and 90th percentiles. The open circles indicate the average values. The scatter plots
596 represent the data distributions. Mann–Whitney *U* tests were conducted for the pairwise
597 comparisons between angiosperms and conifers: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

598

599 **Figure 2**

600 Difference in the metabolite concentrations of the orthodox photorespiratory pathway between
601 the leaves of angiosperm trees and those of conifers. The values adjacent to each metabolite
602 species represent the ratio of the two-year average concentration of the 13 conifer species to that
603 of the 14 angiosperm tree species (Supplemental Table S1). The measured metabolites are
604 boxed, and those that showed statistically significant differences between the two taxonomic
605 groups are shown in color based on the calculated ratios (blue, decrease; orange, increase). Fd^(ox),
606 oxidized ferredoxin; Fd^(red), reduced ferredoxin; 2-OG, 2-oxoglutarate; OAA, oxaloacetate;
607 OH-Pyr, hydroxypyruvate; 2-PG, 2-phosphoglycolate; 3-PGA, 3-phosphoglycerate; RuBP,
608 ribulose-1,5-bisphosphate. The enzymes are indicated by circles (peroxisome-targeting enzymes
609 are denoted in white letters in dark-green circles): CAT, catalase; GLO, glycolate oxidase; GDC,
610 glycine decarboxylase complex; GGAT, glutamate-glyoxylate aminotransferase; GLYK,
611 glycerate kinase; GOGAT, glutamate-2-OG aminotransferase; GS, glutamine synthetase; HPR,
612 hydroxypyruvate reductase; MDH, malate dehydrogenase; PGLP, phosphoglycolate
613 phosphatase; SGAT, serine-glyoxylate aminotransferase.

614

Figure 3

Heatmap visualization of the leaf photorespiratory metabolites labeled with a stable isotope of carbon (^{13}C). Ser (1.6 or 3.2 mM) was prepared so that its C3 position was labeled with ^{13}C (^{13}C -Ser) and was then fed to the detached shoots of an angiosperm tree (*Populus nigra*) (A) and those of a conifer (*Cryptomeria japonica*) (B) via the transpiration stream. As a control, unlabeled standard Ser (uSer) feedings were also carried out. The feedings were conducted over 20 or 50 min under illumination in an environmentally controlled growth cabinet. The row displays the metabolite species; the column displays different treatments. The brightness of the red color corresponds to the magnitude of the increases in the relative abundance of the isotopologue, which had a single ^{13}C atom for each metabolite (Student's *t*-test; $P < 0.05$ between the ^{13}C -Ser and uSer feedings); the yellow color indicates the absence of significant differences. The number of shoot samples used in each treatment per species was 3–4 (Supplemental Table S2).

Figure 4

The activities of three enzymes, i.e., hydroxypyruvate reductase (HPR), catalase (CAT), and glycolate oxidase (GLO), were determined in the leaves of 14 angiosperm tree (Ang) and those of 13 conifer (Con) species grown in a field site. The activities were expressed per unit soluble protein content. The left and right panels represent samples collected in 2019 and those collected in 2020, respectively. The central box in each box plot represents the interquartile range and median; the whiskers indicate the 10th and 90th percentiles. The open circles indicate the average values. The scatter plots represent the data distributions. Mann–Whitney *U* tests were conducted for pairwise comparisons between conifers and angiosperms: *, $P < 0.05$; **, $P < 0.01$.

Figure 5

641 Differences in the gene expression and protein sequences of catalase (CAT) between
 642 angiosperms and conifers. A, Relative mRNA expression levels of the *CAT* genes in the leaves
 643 and roots of an angiosperm tree (*Populus nigra*) (left, *PnCAT1-3*) and a conifer (*Cryptomeria*
 644 *japonica*) (right, *CjCATA* and *CjCATB*) saplings. The open and hatched bars represent the leaves
 645 and roots, respectively. The expression levels of the *CAT* genes were normalized against the
 646 expression level of the actin-encoding genes. Mean $\pm$ SD, $n = 3$ biological replicates. B,
 647 Alignment of the amino acid sequences of the CAT proteins of *P. nigra* with those of *C.*
 648 *japonica*. Identical amino acid regions are indicated in gray. The region of the peroxisomal
 649 targeting signal (PTS), which has been implicated in the regulation of the import into
 650 peroxisomes, is boxed. C, Weblogo images of the PTS sequences of the CAT proteins for
 651 angiosperms and conifers. To generate the images, we referred to 51 genes from 18 angiosperm
 652 species, including herbs, grasses, and trees; and 13 genes from nine coniferous species, based on
 653 databases (Supplemental Table S3).

654

655 Figure 6

656 Characteristics of the peroxisome fractions isolated from the leaves of an angiosperm tree
 657 (*Populus nigra*, *Pn*) and from the leaves of a conifer (*Cryptomeria japonica*, *Cj*) using the
 658 Percoll/sucrose density gradient method. $C_{\text{chloro.}}$, chloroplast contamination index; $C_{\text{mito.}}$,
 659 mitochondrial contamination index; L_{CAT} , peroxisomal localization index of catalase enzymes in
 660 the isolated peroxisome fractions. Mean $\pm$ SD from three-to-four independent experiments
 661 (Table 1). The values of n.d. in Table 1 were assumed to be zero for the mean calculations.

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