High Glycolate Oxidase Activity Is Required for Survival of Maize in Normal Air

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A mutant in the maize (Zea mays) Glycolate Oxidase1 (GO1) gene was characterized to investigate the role of photorespiration in C4 photosynthesis. An Activator-induced allele of GO1 conditioned a seedling lethal phenotype when homozygous and had 5% to 10% of wild-type GO activity. Growth of seedlings in high CO2 (1%–5%) was sufficient to rescue the mutant phenotype. Upon transfer to normal air, the go1 mutant became necrotic within 7 d and plants died within 15 d. Providing [1-14C]glycolate to leaf tissue of go1 mutants in darkness confirmed that the substrate is inefficiently converted to 14CO2, but both wild-type and GO-deficient mutant seedlings metabolized [1-14C]glycine similarly to produce [14C]serine and 14CO2 in a 1:1 ratio, suggesting that the photorespiratory pathway is otherwise normal in the mutant. The net CO2 assimilation rate in wild-type leaves was only slightly inhibited in 50% O2 in high light but decreased rapidly and linearly with time in leaves with low GO. When go1 mutants were shifted from high CO2 to air in light, they accumulated glycolate linearly for 6 h to levels 7-fold higher than wild type and 11-fold higher after 25 h. These studies show that C4 photosynthesis in maize is dependent on photorespiration throughout seedling development and support the view that the carbon oxidation pathway evolved to prevent accumulation of toxic glycolate.

The oxidation of glycolate to glyoxylylate in higher plants is catalyzed by glycolate oxidase (GO; EC 1.1.3.15), an FMN-containing protein (Zelitch and Ochoa, 1953). The enzyme is located in the peroxisome and performs an essential step in the operation of the oxidative photorespiratory cycle accompanying photosynthetic CO2 assimilation in C3 plants (Tolbert, 1971, 1997). Glycolate arises from 2-phosphoglycolate, which is produced together with 3-phosphoglycerate by ribulose bisphosphate reacting with O2 as catalyzed by Rubisco (Bowes et al., 1971; Ogren, 2006) in the bundle sheath (BS) cell of C4 plants (Edwards and Walker, 1983). Dephosphorylation of 2-phosphoglycolate yields glycolate that is transported into the peroxisome. In the peroxisome, GO oxidizes glycolate to produce glyoxylylate. Transamination of glyoxylylate generates Gly that is converted to Ser and CO2 in the mitochondrion (Tolbert, 1971). The Ser gives rise to glyceraldehyde, which is ultimately converted to 3-phosphoglyceric acid in the mesophyll (M) cell chloroplast (Usuda and Edwards, 1980). Thus, the photorespiratory pathway (Fig. 1) is a futile process that diverts energy to refixation of photorespired CO2, thereby lowering the quantum efficiency of net carbon assimilation (Zelitch, 2001).

In C3 leaves, production of photorespiratory CO2 decreases net CO2 assimilation by about 25% at 25°C, and photorespiration increases greatly relative to CO2 assimilation at higher temperatures (Peterson, 1983; Hanson and Peterson, 1986). In C4 plants such as maize (Zea mays), a carbon shuttle increases local CO2 concentrations in the BS chloroplasts, where Rubisco is located, to levels more than three times higher than those found in M cells of C3 plants. As a result, photorespiration is greatly reduced in C4 tissues (Hatch, 1971; Jenkins et al., 1989; Dai et al., 1993; Laisk and Edwards, 1998).

Several studies point to a low, yet finite, rate of photorespiration in maize. To estimate photorespiration in maize leaves, Zelitch (1973) compared the initial rate of glycolate accumulation in leaf tissue in high light in the presence of an inhibitor of GO, α-hydroxysulfonate, and found that glycolate accumulation was 10% as great in maize as in tobacco (Nicotiana tabacum; C3). Because no mechanism for the metabolism of glycolate is known without its first being converted to glyoxylylate, this demonstrated that the rate of CO2 production by photorespiration was no more than 5% of the net CO2 assimilation rate (A) in C4 photosynthesis. Additional studies that monitored 18O2 and 13CO2 exchange and estimates from electron transport suggested a similar photorespiratory rate in maize of 2% to 7% of A (Volk and Jackson, 1972; de Veau and Burrus, 1989; Laisk and Edwards, 1998). Experiments with maize leaves in high light showed that with increasing O2 level, there was
some decrease in $A$, and in limiting light a decrease in
the maximum quantum yield was observed, further
indicating that some photorespiration occurred (Dai
et al., 1993).

A number of conditional mutants with lesions in
different steps of the photorespiratory pathway were
first obtained by Ogren and coworkers in Arabidopsis
($Arabidopsis thaliana$; Ogren, 1984, 2006) and later by
others in barley ($Hordeum vulgare$; Blackwell et al., 1988)
and tobacco (McHale et al., 1988). In these studies,
mutants were identified that could survive in high CO$_2$,
when photorespiration was blocked, but succumbed in
normal air. Indeed, analyses of metabolite accumula-
tion in these mutants provided biochemical evidence
for a photorespiratory cycle (for review, see Reumann
and Weber, 2006). However, no mutant lacking $GO$ was
ever recovered by this method of selection. The pres-
ence of five distinct $GO$ loci in the Arabidopsis genome
likely accounts for the failure to recover conditional
$GO$ mutants in previous screens.

Transgenic tobacco plants were generated that had
reduced $GO$ activity through cosuppression of the
endogenous $GO$ (Yamaguchi and Nishimura, 2000). These plants contained variable levels of $GO$ activity
that decreased during plant development, with some
plants ultimately having as little as 20% of wild-type
activity. Transgenic plants with greatly reduced levels
of $GO$ exhibited dramatically reduced photosynthetic
electron transport rates in high light, whereas plants
with moderate levels of $GO$ were less affected.

In this report, we describe the isolation and charac-
terization of a maize $GO$ mutant caused by an $Activator$
($Ac$) insertion in $GO1$ ($go1-m1::Ac$). Using $Ac$ as a
molecular tag, the $GO$ gene was cloned and sequenced.

In addition, mutant seedlings were characterized un-
der both high and low CO$_2$ conditions. Under high CO$_2$
conditions, $GO$ deficiency had no visibly adverse ef-
fects on plant growth. However, $A$ was rapidly and
linearly diminished in mutants in high light and high
O$_2$. When such mutant plants were transferred from
growth in high CO$_2$ to normal air in the light, glycolate
accumulated linearly for 6 h to levels 7-fold greater than
the initial concentration. Plants greatly depleted in $GO$
activity were nonviable when grown at ambient CO$_2$
concentrations. We therefore conclude that photores-
piration plays a vital role in $C_4$ photosynthesis.

**RESULTS**

**Identifying a Maize $GO$ Mutant**

The $GO$ mutant was first identified in sandbench
screens of $Ac$-mutagenized families (Kolkman et al.,
2005). A single family was identified that segregated a
seedling lethal phenotype in 21 of the 71 seedlings
examined, suggesting that the mutation was due to
disruption of a single gene ($\chi^2 = 0.23; P = 0.63$). DNA-
blot analysis was used to identify an $Ac$-containing, 3.2-
kb EcoRI fragment that cosegregated with the mutant
phenotype (data not shown). Approximately 800 bp of
DNA flanking this sequence was recovered using an
inverse PCR technique as described in “Materials and
Methods.” To recover additional sequences flanking the
$Ac$ insertion, we exploited the somatic instability of
the active $Ac$ insertion to selectively amplify $Ac$ junction
fragments that were generated through local insertions.

**Figure 1.** Schematic diagram of $C_4$ photosynthetic CO$_2$ assimilation in maize. The incoming atmospheric CO$_2$
reacts (1) with phosphoenolpyruvate (PEP) to form oxaloacetate (OAA) in the M cell. The OAA is reduced (2) to malate through the NADP-
aldehyde dehydrogenase reaction, and the malate is converted to pyruvate and CO$_2$ by the NADP-malic enzyme (3) in the
chloroplasts of BS cells. The released CO$_2$ combines with ribulose bisphosphate (RuBP) through the Rubisco reaction (4) to form
phosphoglycerate (PGA). The resulting elevated CO$_2$ level inhibits, but does not eliminate, the oxygenase reaction (5) of Rubisco.
Photosynthetic products are likely generated from incomplete suppression of Rubisco oxygenase activity in BS chloroplasts. A
specific phosphatase in the chloroplast (6) converts the resulting P-glycolate to glycolate. The glycolate is transported to the
peroxisome where $GO$ activity (7), when present at high levels, creates glyoxylate. A series of aminotransferase and
decarboxylation reactions leads to the production of Gly and Ser with the release of photosynthetic CO$_2$. Note that two $Gly$
combine to produce one Ser and one CO$_2$. When $GO$ activity is low or absent, the glycolate, which has no other means of being
metabolized, will accumulate at rates equal to the rate of P-glycolate formation. For further details, refer to the introduction.

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enabled us to recover additional sequence information 1 kb upstream and 1 kb downstream of the original Ac insertion site. This confirmed that the Ac element inserted at the locus was active and enabled us to assemble a contig of approximately 2 kb flanking the Ac insertion site.

The Ac Insertion Is Located in the GO1 Locus

Genomic sequences flanking the Ac insertion were used to search GenBank and matched genomic DNA (AC187560.5) and mRNA (AY108197). Further BLAST searches indicated the Ac insertion was in exon 7 of a putative GO gene (Fig. 2A). We reasoned that the mutant phenotype (necrosis and seedling death) was likely caused by accumulation of glycolate and that it may be rescued under high CO₂ conditions where photorespiration is minimal. In an attempt to rescue the seedling lethality of homozygous go1 mutants, seedlings were grown under high CO₂ conditions (1% to 5% CO₂ in air). Under these conditions, the growth and appearance of the mutants could not be distinguished visually from either the heterozygous or homozygous wild-type individuals (Fig. 3). Seedlings were routinely grown this way for 30 to 60 d. Soon after germination in high CO₂, the phenotype of an individual plant was established using a sensitive GO assay carried out on extracts prepared from discs cut from the first leaf. Later, this phenotype was confirmed on extracts prepared from second or third leaves. As shown in Figure 2B and Table I, seedlings homozygous for the Ac insertion allele showed minimal GO activity. Extracts from heterozygous individuals exhibited intermediate capacities to oxidize glycolate (Table I), indicating that a single functional copy of GO is insufficient to completely rescue the defect in GO activity. Heterozygote plants appear phenotypically normal, complete a full-life cycle, and are not underrepresented among segregating families. Thus, GO activity of heterozygote individuals is sufficient for plant health in normal air under the growth chamber conditions employed here. Plants with the highest GO activities were homozygous for the functional GO1 allele (430-bp band in Fig. 2B). As shown in Figure 2C, wild-type plants accumulated GO mRNA in contrast to the homozygous mutant plants with the low GO activity, consistent with the

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**Figure 2.** Molecular analysis of the maize go1::Ac allele. A, Representation of the maize GO locus (on BAC clone AC187560.5) and location of the Ac insertion. Black boxes signify exons (numbered), while white boxes denote intron sequences. Enlarged region depicts locus from exon 6 through 8 detailing the insertion of an Ac element in the beginning of exon 7. Arrows depict oligonucleotide primers in genomic and Ac-DNA that amplify wild-type sequences (430-bp fragment) or Ac-junction fragments (209 bp). B, Genotypic characterization by PCR analysis of maize seedlings displaying a high or moderate GO activity (lanes 2–14) or low GO activity (lanes 15–23). C, GO gene expression. Transcripts from maize seedlings: wild-type (lanes 1 and 5), heterozygote (lanes 2 and 6), or mutant (lanes 3, 4, 7, and 8) seedlings analyzed by reverse transcription-PCR for GO expression (lanes 5–8) or actin expression (lanes 1–4). Ac transcripts amplify a 436-bp fragment and GO transcripts amplify a 236-bp fragment.

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**Figure 3.** Appearance of maize seedlings with wild-type GO activity (WT), intermediate GO activity, or low GO activity upon transfer from high CO₂ to normal air. The GO activity was 4.44, 2.46, and 0.35 μmol glyoxylate formed (g fresh weight)⁻¹ h⁻¹ for the three plants, respectively. The size bar on the left represents a height of 18 cm. A, After growth for 14 d in high CO₂ when first transferred to normal air. B, After subsequent growth for a further 15 d in normal air.
severe mutant phenotype of the homozygous insertion. When go1 mutant seedlings were transferred from high CO2 to normal air, leaves became necrotic within 7 d, and the seedlings died within 15 d under either growth chamber or greenhouse conditions (Fig. 3B). Taken together these data indicate that the seedling lethality of the mutants is due to the cytotoxic accumulation of glycolate in homozygous individuals due to an Ac insertion in the maize GO1 gene. We therefore named the mutant allele go1-m1::Ac. A phylogenetic analysis of known GOS from Oryza sativa and Arabidopsis reveals two separate protein subgroups (Fig. 4A) in which the maize GO1 (derived from mRNA PC0065133 gb AY108197) is most similar to proteins encoded by Os07g0152900 (91.0%/96.8% identity/similarity) and At3g14415 (83.7%/94.8%; Fig. 4B).

CO2 Assimilation in Low GO Mutant Is Strongly Inhibited in High O2

To examine the consequences of the loss of GO activity on A, wild-type and go1 mutant leaf tissue was examined under low (1%) and high (50%) O2 conditions (Table II). Representative results are shown in Figure 5. Values of A attained relatively stable levels after 20 min in high light and low O2 (Fig. 5). The average value of A attained after 40 to 50 min in 1% O2 was lower for the mutant, but this effect was not statistically significant (P > 0.05; Table II). A similar trend was noted for effect of genotype on intrinsic quantum yield of PSII (Fv/Fm in Table II). Though not visible by eye, leaf-to-leaf variation in Fv/Fm was unusually high among mutant leaves, consistent with the occasional occurrence of mild cytotoxic symptoms. An immediate 3% to 4% reduction in A occurred upon increasing the O2 level from 1% to 50% for both genotypes (Fig. 5). During the remainder of the exposure to 50% O2, the magnitudes of A declined on average by 16% (10% in the example illustrated in Fig. 5) for wild type. A similar inhibition of A at high O2 was observed in maize leaves by Dai et al. (1993). In go1 mutants, A declined by an average of 93% (92% in the example given in Fig. 5) relative to high CO2 conditions. The decline in A in the go1 mutants in 50% O2 was essentially linear (Fig. 5), and A was almost completely abolished after 50 min at this higher O2 level.

Table I. Distribution of GO activity in segregating populations of maize plants grown from seeds of selfed mutant heterozygous plants with decreased GO activities

| Plant Material | GO Activity | µmol glyoxylate formed (g fresh weight)−1 h−1 |
|----------------|-------------|---------------------------------------------|
| Low GO         | 0.66 ± 0.08 | (n = 39)                                    |
| Intermediate GO| 2.86 ± 0.14 | (n = 88)                                    |
| Wild-type GO   | 6.00 ± 0.30 | (n = 65)                                    |

*GO activity was determined on extracts of leaf disks from plants grown in 1% to 5% CO2 in a chamber in a growth room as described in "Materials and Methods." Samples were taken from 192 plants in seven separate growth experiments. The mean activity values are given ± SE.
go1 Mutants Do Not Display a General Disruption in the Photorespiratory Cycle

To examine the consequences of a disruption of GO activity on additional reactions of the photorespiratory cycle, go1 mutants were further characterized by providing [1-14C]glycolate to leaf discs in darkness and measuring the 14CO2 produced (Table III). These results are consistent with parallel assays made on leaf extracts showing that go1 mutants do in fact have a low but measurable activity compared with intermediate and normal plants. An important later step in photorespiration, after oxidation of glycolate, involves the conversion of two Gly to Ser and CO2 (Fig. 1). Leaf discs taken from go1 mutants were tested to determine whether any later step in the pathway was also affected by supplying [1-14C]Gly in darkness. Table IV shows that the rate of formation of [14C]Ser and 14CO2 was similar for all three genotypes. The mean ratio of CO2 produced:Ser formed was 1.02, close to the expected stoichiometry, and an ANOVA showed there was no significant difference in the mean ratios among the three genotypes. Thus, plants with low GO activity were not likely altered in their ability to otherwise complete metabolism of photorespiratory Gly.

Glycolate Accumulates in Normal Air in the Low GO Mutant

Though considerable phosphoglycolate is produced by the Rubisco oxygenase reaction, a high GO activity keeps the free glycolate concentration at low steady-state levels in leaves, and even when glycolate concentration increased greatly in short-term experiments in tobacco treated with an inhibitor of GO, the phosphoglycolate concentration was unaffected (Zelitch, 1965). The low concentration of glycolate is likely due solely to the function of GO, as there is no other known mechanism for metabolizing glycolate without it first being converted to glyoxylate. By means of isotope dilution, a glycolate concentration of 0.471 ± 0.038 (se) μmol/g fresh weight was deduced for maize leaves in light (Jolivet et al., 1985), and by use of HPLC, a value of 1.2 μmol/g fresh weight was obtained (González-Moro et al., 1997). A similar mean steady-state value for wild-type leaves can be obtained from Figure 6 (n = 33) of 0.93 ± 0.12 (se) μmol glycolate/g fresh weight (1.0 cm² leaf area = 14 mg fresh weight).

When seedlings were transferred from growth in high CO2 to normal air under the same growth conditions, glycolate concentration in the leaves increased rapidly and linearly for at least 6 h in go1 mutants to a level of 6.3 μmol glycolate/g fresh weight, a 7-fold increase (Fig. 6). After a total of 25 h in air, the glycolate concentration increased to 10.0 μmol glycolate/g fresh weight, an 11-fold increase over the initial concentration. An increase in glycolate concentration in maize leaves induced by photorespiratory inhibitors was accompanied by a decrease in A (González-Moro et al., 1997), and a level of 4.0 μmol glycolate/g fresh weight was sufficient to completely abolish A. This inhibitory glycolate concentration was reached after about 3 h in normal air in mutant leaves (Fig. 6). The linear increase in glycolate in go1 mutants (Fig. 6) is consistent with the linear loss of A capacity in 50% O2 and high light (Fig. 5).

DISCUSSION

Consistent with lower rates of oxygenation of ribulose bisphosphate, it has often been noted that in C4 plants, the enzymes of the photorespiratory pathway,

| Parameter | Wild Type | Low GO |
|-----------|-----------|--------|
| A (1% O2) | 9.64 ± 1.28 | 7.96 ± 0.37 |
| Initial A (50% O2) | 8.93 ± 0.89 (96.2% ± 0.3) | 7.70 ± 0.37 (96.8% ± 0.6) |
| Final A (50% O2) | 7.91 ± 1.48 (83.9% ± 5.8) | 0.54 ± 0.09 (7.0% ± 1.1) |
| F/Fm | 0.744 ± 0.004 | 0.689 ± 0.029 |

*a A significant difference due to phenotype based on a T test (P < 0.05).*
including GO, are present at much lower levels than in C₃ plants (Edwards and Walker, 1983; Dai et al., 1995). For example, the GO specific activity in C₃ grasses is approximately 10-fold greater than in maize (Ueno et al., 2005), and we have also found (I. Zelitch, unpublished data) that on a leaf area basis, maize has about 10% as much GO activity as the C₃ tobacco leaf. Nevertheless, no mutant has previously been described in a C₃ plant in which GO, or any other gene underlying an enzymatic step of the photorespiratory pathway, was defective. We have shown here that a homozygous disruption of the GO1 gene produces a conditional lethal mutation. This mutant is analogous in its phenotype to other mutants that disrupt enzymatic steps in photorespiration, as previously described for Arabidopsis and other C₃ species (Ogren, 1984, 2006) and shows that a functional photorespiratory pathway is essential for maize seedling development.

As shown in Figure 1, glycolate is an early intermediate following the oxygenation of ribulose-1,5-bisphosphate. To examine the effects of glycolate accumulation on carbon assimilation in maize, González-Moro et al. (1997) excised maize leaves in light and supplied them with inhibitors of photorespiratory enzymes. Following the treatments, leaf glycolate concentrations rose from 1.2 μmol/g fresh weight to 4.0 μmol/g fresh weight, at which time CO₂ assimilation was completely blocked, Rubisco activity decreased, and the ribulose bisphosphate concentration increased. They later showed that adding 10 or 20 mM glycolate to excised maize leaves together with low levels (1 mM) of phosphinothricin, an inhibitor of Gln synthetase, greatly increased the inhibitory effect on CO₂ assimilation within several hours (González-Moro et al., 2003). These inhibitory effects were attributed to the higher leaf glycolate concentrations.

Three isoforms of GO have been identified in maize. Two isoforms are localized to the BS, representing 80% of the total activity, and one to the M cells (Popov et al., 2003). Given the restriction of Rubisco to the BS cells, it is perhaps not surprising that the majority of the GO activity is restricted to this compartment. Several lines of evidence support the contention that the majority of this activity is associated with GO1. First, a single lesion at the GO1 locus eliminates all but 5% to 10% of the activity of wild-type leaves as assayed in leaf extracts (Table I) and shows a low rate of metabolism of [1-¹⁴C] glycolate in intact leaf tissue (Table III). Importantly, this residual activity in the mutant is insufficient to prevent rapid accumulation of glycolate in normal air and seedling lethality. Second, molecular analyses correlated genotype with enzymatic phenotype. Plants with intermediate or high GO activities had at least one wild-type GO1 locus, whereas all plants with low GO activities were homozygous for the Ac insertion in the GO1 locus (Table I; Fig. 2). Third, the maize GO1 is most similar to Oryza and Arabidopsis GO enzymes, which are highly expressed in leaves (Fig. 4B). Although we attempted to examine the cell-specific expression of GO1 in separated BS and M cells, it was not possible to conclusively determine cell-specific expression as the transcript is transcribed at a lower rate or rapidly degraded during the isolation of maize M cell protoplasts (data not shown).

As we have discussed, photorespiration in maize is normally low, but our results confirm that it is not

| Table III. Metabolism of [1-¹⁴C]glycolate by maize leaf discs in darkness in a segregating mutant population with varying GO activities |
|--------------------------------------------------|
| The mean GO activities assayed from leaf disc extracts are given for each plant material ± s (n = 5). Enzymatic ¹⁴CO₂ production during the 2-h incubation is likewise reported as the corrected count rate (n = 3). |
| Plant Material | GO Activity | Enzymic ¹⁴CO₂ Released |
|----------------|-------------|------------------------|
| Low GO         | 0.19 ± 0.07 | 252 ± 28               |
| Intermediate GO| 3.44 ± 0.60 | 8,400 ± 2,160          |
| Wild-type GO   | 5.35 ± 1.00 | 7,300 ± 3,790          |

| Table IV. Metabolism of [1-¹⁴C]Gly to ¹⁴CO₂ and [¹⁴C]Ser in darkness by maize leaf discs in a segregating mutant population with varying GO activities |
|-------------------------------------------------------------------------------------------------------------------------------------|
| The [¹⁴C]Gly and [¹⁴C]Ser were separated by HPLC as described in “Materials and Methods.” The mean values are given for each plant material ± s (n = 3). |
| Plant Material | ¹⁴CO₂ Released | [¹⁴C]Ser Formed | ¹⁴CO₂ to [¹⁴C]Ser |
|----------------|----------------|----------------|-------------------|
| Low GO         | 23,900 ± 2,410 | 21,300 ± 1,260 | 1.12              |
| Intermediate GO| 22,900 ± 1,730 | 22,400 ± 2,080 | 1.02              |
| Wild-type GO   | 25,000 ± 5,340 | 29,200 ± 7,510 | 0.86              |

*One-way ANOVA indicated no significant effect (P = 0.43) of GO phenotype on the ¹⁴CO₂ to [¹⁴C]Ser ratio. The mean ratio calculated across all phenotypes was 1.02 (s = ± 0.07; n = 9).
entirely absent, so we must conclude that the CO$_2$-concentrating mechanism in the BS cells does not completely suppress Rubisco-catalyzed oxygenation of ribulose bisphosphate, the primary source of glycolate (Figs. 1 and 6).

This conclusion is consistent with previous studies (Dai et al., 1995; González-Moro et al., 1997, 2003) that suggested photorespiration is operational in maize. In 50% O$_2$ and high light, we also observed that A declined rapidly and linearly with time in the low GO mutant (Table II; Fig. 5). Moreover, and despite residual low levels of GO activity in GOI mutants, there are indications of chronic glycolate toxicity even under high CO$_2$ conditions used to rescue these mutants. This is not surprising when considered in the context of the Rubisco mechanism (see below). We note the modest tendency toward lower values of A in 1% O$_2$ and especially $F_v/F_m$ in the mutants (Table II; Fig. 5). The latter is a sensitive indicator of stress. This suggests that even minute traces of glycolate production exert cumulative detrimental effects that become visible only in the later stages.

The reaction catalyzed by GO is the only known route of glycolate metabolism in higher plants (see below). We note the modest tendency toward lower values of A in 1% O$_2$ and especially $F_v/F_m$ in the mutants (Table II; Fig. 5). The latter is a sensitive indicator of stress. This suggests that even minute traces of glycolate production exert cumulative detrimental effects that become visible only in the later stages.

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ase reaction occurs. The presence of the photosynthetic pathway is even more imperative insofar as the oxygenase activity of Rubisco is ubiquitous (Jordan and Ogren, 1981) and is, in effect, an intrinsic feature of the interaction of the enzyme with ribulose bisphosphate (Lorimer and Andrews, 1973). Thus, glycolate will inevitably be synthesized if O$_2$ is present. Regardless of the mechanisms of glycolate toxicity, the necessity of avoiding glycolate accumulation presents sufficient selective pressure to account for the universal presence of the photosynthetic pathway in oxygenic photosynthesis. This is consistent with initial findings demonstrating lethal effects of photosynthetic pathway lesions in Arabidopsis (Ogren, 2006). This article offers strong genetic and biochemical support for the importance of photorespiration in maintaining low (nontoxic) levels of glycolate.

MATERIALS AND METHODS

**Ac Mutagenesis and Cloning of GO1**

The go1 mutant was identified in sandbench screens of lines segregating newly transposed Ac elements. To identify an Ac-containing restriction fragment polymorphism, genomic DNA was fractionated using EcoRI and resolved on a 1% agarose gel. A 3.2-kb Ac-containing restriction fragment that cosegregated with the mutant phenotype was identified using the internal 700-bp EcoRI/HindIII fragment of Ac as a molecular probe. Inverse PCR reactions were performed using the iPCR-1 protocol as previously described (Kolkman et al., 2005) to amplify an approximately 800 bp of DNA flanking the Ac insertion. Ac casting reactions were conducted using two rounds of PCR with the Ac-specific primers Tbp34, Tbp35, JGPr, and JGcp3 as previously described (Singh et al., 2003). GO-specific primers used include: GO 3

**Genotype Analysis**

Genomic DNA from leaves was prepared according to Dellaporta (1993). RNA isolation was carried out using TRIzol (Invitrogen) and reverse transcription of RNA with a RCM-RACE kit (Ambion) according to the manufacturer’s recommendations. The oligonucleotide primers ZGO1 and ZGO2 flank the Ac insertion site and amplify a 430-bp DNA fragment from wild-type maize genomic sequences and a 236-bp DNA fragment from reverse transcribed wild-type mRNA. Primers ZGO1 and AcGO1 amplify a 209-bp DNA fragment from genomic sequences corresponding to the Ac insertion allele. PCR parameters employed were: 94°C 3 min; (94°C 3 min; 55°C 30 s; 72°C 1 min) 30 times repeated followed by 72°C 7 min.

**Metabolism of [1-14C]Glycolate and [1-14C]Gly by Leaf Tissue**

Radioactive calcium [1-14C]glycolate was obtained from American Radio-labeled Chemicals. It was dissolved in water and first passed through a column of Dowex 50 (H+ form, Bio-Rad) and eluted with water. Potassium glycolate (0.1 mM) was added to make the final concentration 40 mM. A portion was added to a column of Dowex 1 X8, 100 to 200 mesh, anion-exchange resin (Bio-Rad) 0.7 cm in diameter and 6 cm in height. The column was eluted with 10 mL of water, then with 4 mL of 4 v acetic acid, and these fractions were discarded. Glycolic acid was eluted by collection of the next 10 mL of 4 v acetic acid (Zelitch, 1972). Recovery of glycolic acid was 95% to 98%. Portions of the radioactive glycolic acid were placed in microfuge tubes and concentrated to dryness in a rotary evaporator under vacuum at room temperature to remove the acetic acid.

For metabolic experiments, 12.0- cm leaf discs (6.0 cm$^2$) were cut with a punch from a leaf obtained from plants growing in high CO$_2$ and previously identified as having wild-type, intermediate, or low GO activity. The discs were floated on water and transferred to 50-mL Erlenmeyer flasks containing center wells. Potassium [1-14C]glycolate (1.0 mL, 40 mCi, 2.21 × 10$^3$ dpm) was added to each flask, and a control flask omitting leaf discs was used in every experiment. A paper wick moistened with 5% methanolamine was placed in the centerwell to trap 14CO$_2$. The flasks were covered with aluminum foil, closed with a rubber serum stopper, and shaken for 2 h at 20°C. The paper wicks were removed to scintillation vials and the radioactivity determined by scintillation spectrometry.

Experiments on the metabolism of [1-14C]Gly (7.1 × 10$^6$ dpm), were carried out in a similar manner as those with [1-14C]Glycolate. At the end of the experiments, the 14CO$_2$ was determined as before. The flask was filled with water to dilute the radioactive substrate, and the leaf discs were killed by placing them in a Ten Broek homogenizer containing 5 mL of boiling 20% ethanol and kept in a boiling water bath for 5 min. The leaf tissue was ground in the homogenizer, centrifuged, the residue washed with water and centrifuged again, and the combined supernatants further analyzed for radioactive metabolites. A portion of each sample was placed on a Dowex-1 acetate column, and the neutral and basic compounds, including Gly and Ser, were eluted with water. A 1.0-mL portion of each sample was concentrated to 400 μL in a rotary evaporator under vacuum, and 50-μL samples were placed on a HPLC cation exchange Na+ column (PRP X 200, 4.2 × 250 mm, Hamilton) and eluted at 30°C with 25% [NaCl/NaOH 0.05 mM] and 75% water. Ser and Gly eluted separately in this system. Eluates containing each compound were collected in scintillation vials and their radioactivity determined by scintillation spectrometry.
Photosynthesis Measurements

All measurements were conducted using a computer-controlled, dual channel flow-through apparatus previously described (Peterson et al., 2001). The GO phenotypes of the test plants were confirmed by enzyme assay prior to the gas exchange measurements. Plants were first darkened for 12 h to fully reverse photoprotective thermal dissipation processes. Steady-state chlorophyll fluorescence levels ($F_o$ and $F_m$) were then measured for the dark-adapted leaves using a PAM 101 system equipped with an ED101BL emitter-detector unit (H. Walz). The maximum quantum yield of PSII was estimated as $(F_m - F_p)/F_m = (F_o/F_m)$ after correction of measurements for fluorescence emission from PSI (Peterson et al., 2001). Rates of $A$ by attached maize leaves were measured at 360 $\mu$mol m$^{-2}$ s$^{-1}$. The increase in $A$ was recorded continuously in a gas phase $O_2$ concentration of 1% (balance N$2$) for 40 to 50 min. The $O_2$ level was then raised to 50%, and recording of $A$ continued for a similar time period.

**Determination of Glycolate Levels in Leaf Tissue in Normal Air**

Plants were grown in high CO$2$ under temperature- and light-controlled conditions as described above. Plants were kept in normal air in darkness for 1 h. Leaf segments were cut from a leaf (zero time), and the segments were placed in microfuge tubes and centrifuged again. The segments were then maintained for the plants as before but in normal air. Successive segments (2–6 cm$^2$) were cut from the same leaf during the time course, and the segments were placed in microfuge tubes and centrifuged again. The residue was suspended in water and centrifuged. The $O_2$ level was then raised to 50%, and recording of $A$ continued for a similar time period.

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