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Aspects of Glutamate Synthase Activity Deficiency in **Arabidopsis** Metabolism

by

Robert M. Grumbles

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Department of Genetics

EDMONTON, ALBERTA

FALL 1986

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Abstract

The effect of the lack of ferredoxin(fd)-dependent glutamate synthase (GOGAT) activity on the metabolism and growth of **Arabidopsis thaliania** was studied. Because of the plant's small size, the crucifer **Brassica napus** was used as a source material for the isolation and partial characterisation of GOGAT activities from a crucifer. From this characterisation, it was determined that fd-dependent GOGAT was found in both root and leaf tissues and its activity in leaf tissue was affected by light. NADH-dependent GOGAT was also found in both root and leaf tissue, and its activity in the leaf was slightly affected by light. The two enzymes were distinct in their requirements for a reductant source, as isolated fd-dependent GOGAT could not use NADH as an electron donor, and NADH-dependent GOGAT could not use fd. Both enzymes were localised in the plastid fraction of the leaf.

Twenty-eight mutants (**GLUS**) of **A. thaliania** (previously isolated by Somerville and Ogren 1980, Nature 286: 257) were studied genetically, biochemically and immunologically. The mutants were all allelic and all contained cross-reacting material to antibodies raised against spinach leaf fd-dependent GOGAT. In each mutant strain, the fd-dependent GOGAT polypeptide was found to be identical in apparent molecular weight to that of the wild-type polypeptide. In the mutants, only fd-dependent GOGAT was found to be

deficient in activity, NADH-dependent GOGAT activity was normal.

The rate of photosynthetic CO_2 exchange declined in **GLUS** plants in normal air, while the wild-type rate remained unchanged. This decline in photosynthetic CO_2 gas exchange did not correlate with accumulated NH_4^+ produced by mature leaves from mutant plants, nor was there a lack of ribulose 1,5-bisphosphate in mature leaves during the initial inhibition of photosynthetic CO_2 exchange. Also, the activities of key photosynthetic enzymes were slightly reduced at the time when photosynthetic CO_2 exchange declined. The analysis of photosynthetic products recovered after $^{14}CO_2$ fixation suggested that the activity of the photosynthetic carbon reduction (PCR) cycle must be altered, as a higher percent of ^{14}C was recovered in ribulose 1,5-bisphosphate, fructose 1,6-bisphosphate and the sugar monophosphate fraction in the mutant, when compared with the WT strain. The alteration in PCR cycle activity was thought to be a result of an imbalance in the utilization of photosynthetic energy between electron transport and the PCR cycle. It was proposed that certain PCR cycle enzymes were inhibited, causing the decline in photosynthetic CO_2 exchange.

A reversion analysis was carried out with three **GLUS** alleles. The high frequency with which revertants containing near normal levels of fd-dependent GOGAT activity were found

in two of the strains, suggested that these alleles were probably single base pair alterations in the DNA sequence encoding fd-dependent GOGAT protein. From a third allele, no revertants were isolated with a restored fd-dependent GOGAT activity. In studies dealing with revertants isolated from this third allele (CS 254) of the GLUS locus, plants were isolated with a slightly increased chlorophyll and protein level when compared with the GLUS strain after growth in normal air. However, the nature of the specific cause(s) for the apparent increased growth was not determined. It was proposed that the inviability of the GLUS strain in normal air may be due to the reduced photosynthetic rate. Consequently, the photosynthetic apparatus was found to be susceptible to photoinhibition. Photosynthetic CO_2 exchange and electron transport rates were found to decrease faster during stress of the GLUS strain by growth in normal air, than did chlorophyll or protein levels.

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List of Abbreviations

AAN	aminoacetonitrile
ALA	alanine
ASP	aspartate
ATP	adenosine 5'-phosphate
Bicine	N,N-bis(2-hydroxyethyl)glycine
BSA	bovine serum albumin
CO_2	carbon dioxide
Chl	chlorophyll
Cpm	counts per minute
CRM	cross reactive material (immunological)
$^{\circ}C$	degrees centigrade
DEAE	diethylaminoethyl-cellulose
DTT	dithiothreitol
Dpm	disintegrations per minute
E	Einstein (unit of energy)
EDTA	ethylenediaminetetraacetate
EGTA	ethyleneglycol-bis(B-aminoethyl ether)-NN'tetratacetate
FBP	fructose 1,6-bisphosphate
fd	ferredoxin
FBPase	fructose 1,6-bisphosphatase
fw	fresh weight
g	force of gravity
GLN	glutamine
GLU	glutamate
GLUS	glutamate synthase activity deficient strain
GLY	glycine
GOGAT	glutamate synthase (glutamine-2-oxoglutarate aminotransferase)
hr	hour
IRGA	infrared gas analyser
K phosphate	KH_2PO_4/Na_2HPO_4
MSO	methionine sulfoximine
mg/fw	milligrams per fresh weight
min	minute
M	molar
MW	molecular weight
N_2	nitrogen (gas)
NAD	nicotinamide-adenine-dinucleotide
NADP	nicotinamide-adenine-dinucleotide-phosphate
NH_3	ammonia
NH_4^+	ammonium
NO_3^-	nitrate
NP-40	nonidet P-40 (detergent)
OAA	oxaloacetic acid
PGA	3-phosphoglyceric acid
PAR	photosynthetic active radiation
PCO cycle	photosynthetic carbon oxidation cycle
PCR cycle	photosynthetic carbon reduction cycle
PAGE	polyacrylamide gel electrophoresis

PEP	phosphoenolpyruvate
PSI or II	photosystem I or II
P_i	inorganic phosphate
ppm	parts per million
RuBP	ribulose 1,5-bisphosphate
RuBISCO	ribulose 1,5-bisphosphate carboxylase
R5P	ribulose 5-phosphate
s	second
SDS	sodium dodecyl sulfate
SE	standard error
SER	serine
SMP	sugar monophosphate metabolites
TEMED	N',N',N',N',-tetraethylethylenediamine
TCA	tricarboxylic acid cycle
TLC	thin-layer chromatography
Triose-P	dihydroxyacetone phosphate and glyceraldehyde 3-phosphate
Tris	tris(hydroxymethyl)aminomethane
TRIZMA	tris(hydroxymethyl)aminomethane
WT	wild-type ("normal" genetic strain)
2-OXG	2-oxoglutarate

Introduction

The amount of growth of a plant depends on a balance between the energy acquired by photosynthesis and the energy lost through the inhibition of photosynthesis by the environment and by energy loss through respiration. From the time of the emergence of the cotyledons to the [illegible] of its life cycle, the plant derives all its energy from photosynthesis. This process occurs in the chloroplasts, where H_2O is split by light energy to yield O_2 and electrons. The electrons are later donated to inorganic molecules, principally CO_2. The CO_2 is fixed in the metabolism of the Calvin-Benson cycle, also known as the photosynthetic carbon reduction (PCR) cycle. The products of this cycle are the triose phosphates (triose-P): glyceraldehyde-3-P (G3P) and dihydroxyacetone-P (DHAP). In the leaf, triose-P is converted to starch, sucrose, amino acids and other molecules.

Oxygen reduces photosynthetic CO_2 exchange in plants (Bjorkman 1966). This results from the direct and competitive interaction of O_2 at the active site of the enzyme, ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBISCO), which fixes both CO_2 and O_2 (Bowes and Ogren 1972; Laing et al. 1974; Lorimer 1981). As a result of the fixation of one molecule of O_2, one less molecule of CO_2 is

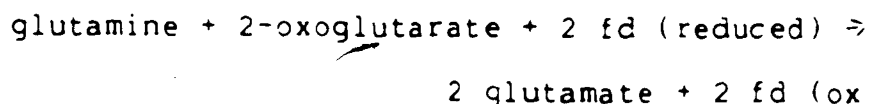
fixed. Also, once an O_2 molecule is fixed by RuBISCO, a conjunctional pathway to that of the PCR cycle is initiated. This other pathway, the photosynthetic carbon oxidation (PCO) or photorespiratory cycle, will release a molecule of CO_2. Therefore, the rate of CO_2 fixation is further reduced as less carbon is available for leaf metabolism. This pathway reduces plant growth for many crop plants, such as wheat and soybean (Bjorkman et al. 1969). Many questions have been raised about whether photorespiration can be altered in a beneficial way, to actually increase yield (Ogren 1978).

In order to address questions about photorespiration, mutant screens were developed to facilitate the isolation of plants with reduced or altered photorespiratory properties (Cannel et al. 1969; Widholm and Ogren 1969). These initial experiments were unsuccessful in isolating plants with altered photorespiratory properties. However, using **Arabidopsis thaliania** a screening procedure was developed from which over 100 mutants, blocked in individual reactions of photorespiratory metabolism, were isolated (Somerville and Ogren 1982a; Somerville 1984). The same screening procedure has also been successful with barley (Kendall et al. 1983). The success of the later screens with **A. thaliania** and barley are due to the fact that the screening procedure was developed to isolate plants lacking a single function. Whereas, the initial screen to find reduced photorespiratory mutants, is dependent on the occurrence of

more than one genetic alteration, as the plants sought after must alter their metabolism in a beneficial way. Mutants isolated from this photorespiratory screen have added to our understanding of photosynthesis by causing a re-evaluation of previous conclusions regarding the metabolic reactions and consequences of the PCO cycle.

One class of mutants (**GLUS**) isolated using the photorespiratory screen with **A. thaliana**, was found to be deficient for ferredoxin-dependent glutamate synthase (fd-dependent GOGAT) activity (Somerville and Ogren 1980a). This is a conditional mutant, since plants deficient for fd-dependent GOGAT activity cannot live in normal air (21% O_2 and 340 ppm CO_2), but do survive in CO_2-enriched air (21% O_2 and 2000 ppm CO_2). In normal air these plants are nearly completely inhibited in photosynthesis. As will be discussed in the next section, this is thought due to the disruption of carbon metabolism in the PCO cycle.

The physiological properties of the **GLUS** mutants also underscored the role of fd-dependent GOGAT activity in leaf NH_4^+ assimilation and for providing glutamate for the transaminase reactions of the cell (Wallsgrove et al. 1983). The enzyme catalyses the following reaction:



glutamine + 2-oxoglutarate + 2 fd (reduced) →

2 glutamate + 2 fd (oxidised)

The glutamate serves as the substrate for glutamine synthetase. Glutamine synthetase and GOGAT work in concert

to assimilate NH_4^+. A GOGAT isozyme which uses NADH as a reductant source also exists in plants (Suzuki and Gadal 1984).

In the current study, a more detailed analysis of the effects of the **GLUS** lesion on the physiology and growth of **Arabidopsis** plants was carried out. In order to provide a context for later experiments investigating the effects of the **GLUS** lesion on cell metabolism, the types of GOGAT activities found in **A. thaliania** and the closely related crucifer **Brassica napus** (rapeseed, canola) were characterized. Secondly, an attempt was made to classify 26 **GLUS** mutants with regards to the molecular defects leading to the loss of enzyme activity. In this approach an antibody against fd-dependent GOGAT was used, to determine the level of fd-dependent GOGAT protein present in the activity-deficient plants. The third part of this thesis concerned an investigation into the cause(s) of the inhibition of photosynthesis in **GLUS** plants. Various proposals have been put forth to explain the inhibition of photosynthesis observed during CO_2 fixation in normal air. These include: a) NH_4^+ accumulation to toxic levels (Somerville and Ogren 1980a), b) the blockage of carbon metabolism in the PCO cycle, resulting in a severe depletion of PCR cycle intermediates (C. R. Somerville and Ogren 1980b; S. C. Somerville and Ogren 1983), c) the inactivation of key enzymes in the PCR cycle because of altered chloroplast metabolism in response to the reduced re-entry of carbon

from the PCO cycle (Creach and Stewart 1982), d) the buildup of glyoxylate and subsequent inhibition of RuBISCO (Cook and Tolbert 1982). Each of these proposals was examined by comparing the metabolism of GLUS plants with that of normal (WT) A. thaliania plants. Another aspect of this thesis has been to isolate revertant plants with a GLUS background which would grow in normal air. By this approach it was envisioned that other cellular functions would be mutagenised and their contribution to the GLUS phenotype assessed. Mutagenised plants did, indeed, yield progeny plants which were capable of growth in normal air.

Background

Photochemistry

The primary events of photosynthesis occur within and on the thylakoid, which is the involuted membrane of the chloroplasts. These membranes contain an array of chlorophylls and when particles of light (quanta) strike the membranes of the chloroplast, the chlorophylls become excited. The energy of the excited state can be released as heat, re-emitted as light (fluorescence) or be used for photochemical reactions (Govindjee 1982). Hill (1937) showed that when a cell-free preparation from green leaves was illuminated O_2 evolved if a suitable electron acceptor was present. It was later shown that the electrons necessary for

the excited chlorophyll states came from water (Radmer and Ollinger 1980). The absorbed light energy results in a rapid resonance transfer of electrons to the photosynthetic reaction centers. From the reaction centers, this excited energy is transferred into photosynthetic electron flow via specific carriers within the thylakoid membrane.

There are two photosystems (PSI and PSII) which are asymmetrically arranged across the thylakoids. Each has a unique energy spectra for excitation by light. Both photosystems consist of a separate complex of proteins and chlorophylls. The role of most of the associated chlorophylls is to absorb light and channel the resulting excitation energy to a reaction center. Such a center consists of one or a few chlorophyll a molecules present in the photosystem (Junge 1977). The proteins within the complex of a photosystem possibly orient and space the chlorophylls within the complex, so that light can be absorbed and efficiently be transferred. In higher plants these complexes are also known as the light-harvesting chlorphyll a / b complexes. The transfer of energy appears to be complex and if too much energy is absorbed by one photosystem, then possible cation re-distribution and specific phosphorylations of certain proteins may help balance the distribution of energy within the photosystems (Allen et al. 1981; Barber 1982). Without the array of chlorophylls around the reaction centers, photosynthetic electron transport would be almost nonexistent under light

intensities less than full sunlight.

Essentially, through redox states the reaction centers have transduced light quanta into the energy of electron trânsfer (Junge 1977). The reaction center chlorophyll donates an electron to nearby molecules. In higher plants it is believed that a pheophytin receives an electron from PSII, which then quickly transfers the electron to a plastoquinone (PQ). The acceptance of an electron by a PQ also means that a proton (H^+) is received by the PQ molecule. The electron and H^+ are shuttled across the thylakoid membrane via the PQ molecule. The PQ is then oxdised by a cytochrome b_6-f complex. The f cytochrome is actually a "c-type" cytochrome and electron transfer is via a complex as in other c cytochromes (Barber 1984). The transfer consists of two consecutive single electron reactions to a plastocyanin (Haehnel 1984). The plastocyanin then reduces PSI (P700). Light energy is absorbed by PSI and via the reduction of ferredoxin, a $NADP^-$ is reduced.

As described above, a H^+ gradient develops across the thylakoid memebrane as a result of the noncyclic transfer of electrons from H_2O and through the PQ pool of electron carriers. Using radioactive methylamine (which freely permeates a membrane in response to a pH gradient) as an indicator of H^+ uptake by thylakoids from the surrounding media, Portis and McCarty (1976) showed that the H^+ concentration in the internal side of the thylakoids is

proportional to the rate of electron transport. Further, as H^+ accumulates, the rate of electron transport becomes inhibited. They showed also that ATP synthesis was proportional to internal H^+ concentration. The ATP was synthesized by the H^+-translocating ATPase (Ort and Melandri 1982). Additional work by Davenport and McCarty (1984) with various photosynthetic electron transport inhibitors, suggested (also considering the H^+ released by the oxidation of H_2O) that 2 H^+ are transferred per electron moving through both photosystems and that 3 H^+ are transported per ATP molecule synthesized. The conclusion drawn from these studies and similar ones (reviewed by Ort and Melandri 1982), point to the fact that the thylakoid membrane serves as a bulk insulator of H^+ and that this gradient between the internal and external phases can be used for the synthesis ATP.

Briefly, since only 3 ATP molecules seem to be synthesized per 2 electrons transported (Davenport and McCarty 1984) and photosynthetic CO_2 exchange occurs via the PCR cycle (see below), the ratio of ATP to electron transfer is close to the 3 ATP and 2 NADPH required for photosynthetic fixation of one molecule of CO_2 (Bassham 1965). Any slight alteration in a plant's capability to synthesize ATP or transfer electrons (or affect the ratio of their respective synthesis), would be deleterious to the process of CO_2 fixation. However, processes other than noncyclic electron transport may contribute to the synthesis

of ATP and electron transfer.

Another process to be considered is ferredoxin dependent photoreduction of O_2 (Arnon and Chain 1977; Furba and Badger 1983; Hosler and Yocum 1985). Using isolated thylakoids, light and in the presence of ferredoxin, O_2 uptake was stimulated and independent ofnoncyclic electron transfer. In addition, this process yielded ATP. NADPH can also be used as reductant source for this ferredoxin dependent cyclic photophosphorylation (Arnon and Chain 1977). Conceivably, if ATP synthesis via noncyclic electron transfer is inhibited, any NADPH present can be used to generate "extra" ATP. The role of these other processes for generating ATP and the exact number of ATP produced during noncyclic transport seems to vary with the particular thylakoids prepared for experimentation. The role of these varied processes in the synthesis of ATP **in vivo** is far from being understood (Ort and Melandri 1982).

Photosynthetic CO_2 Assimilation

The NADPH and ATP generated by the photochemical processes of photosynthesis are utilised by the PCR cycle for the fixation of CO_2. Initially, ribulose 1,5-bisphosphate (RuBP) and CO_2 react at the active site of RuBISCO to form 3-phosphoglycerate (PGA). The biochemical steps involved in the assimilation of CO_2 were elucidated by employing radiolabeling techniques and paper chromatography.

For these experiments algal cells were exposed to $^{14}CO_2$ for predetermined time periods and then quickly killed (Benson et al. 1950; Bassham 1965). The metabolites of the cells were extracted and then separated by paper chromatography in order to isolate and identify the products of CO_2 fixation.

The first radioactive product detected was PGA. The synthesis of one triose-P requires one ATP and one NADPH molecule. The reactions of the PCR cycle also serve to re-arrange triose-P carbon into the 5 carbon molecule, ribulose-5-phosphate (R5P). R5P requires a further ATP to regenerate the substrate of CO_2 fixation, RuBP. The PGA is converted to triose-P and is exported from the PCR cycle for the synthesis of starch, sucrose and other compounds, such as amino acids (Lilley 1983).

A role for RuBISCO in CO_2 assimilation was deduced from the inverse relationship between the labeling of PGA and RuBP, along with the mechanistic constraints required to explain the ^{14}C labeling of PGA in the C-1 position of the molecule (Wilson and Calvin 1955; Jakoby et al. 1956; Bassham 1965). Furthermore, isolated chloroplasts have been shown to possess the ability to fix CO_2 and that RuBISCO was localised in the chloroplast (Jensen and Bassham 1966). Additional support for the requisite role for RuBISCO in photosynthetic CO_2 assimilation was obtained from a **Chlamydomonas reinhardii** mutant lacking RuBISCO which was unable to fix CO_2 (Levine and Togasaki 1965).

As a key enzyme of the PCR cycle, RuBISCO is

biochemically regulated (Lilley 1983). When a leaf is first illuminated, photosynthetic CO_2 assimilation does not begin immediately. There is a lag period before CO_2 fixation reaches its maximum rate. When the maximum CO_2 fixation rate is reached, RuBISCO activity is considerably higher than in the dark. As light quanta were absorbed by the photochemical apparatus, H^+ was removed from the stroma and Mg^{2+} released into the stroma (Werden et al. 1975). The increase in pH from 7.2 to 8.0, along with the rise in Mg^{2+} levels, activated RuBISCO (Bassham et al. 1968; Lorimer et al. 1976). It is believed that in the presence of CO_2, the rise in stromal pH and Mg^{2+} levels induce a conformational change in the enzyme, which allows it to be catalytically active (Lorimer 1981). CO_2 also activates RuBISCO by binding to an essential lysine residue to form a carbamate (Lorimer and Miziorki 1980). In fact, the level of CO_2 present in the environment and the intensity of light impinging on the leaf have been shown to regulate the activation level of RuBISCO (Machler and Nosberger 1980; Perchorowicz et al. 1981; Somerville et al. 1982). However, it is still unclear how full activation of RuBISCO occurs and how the activation state is maintained in the leaf. In vitro, CO_2, Mg^{2+} and a pH of 8.0 will not maintain a fully activated state (Lorimer et al. 1976). It has been shown that various phosphorylated metabolites affect the activity of RuBISCO by directly binding to the catalytic site of the enzyme, and they

possibly help to maintain an active enzyme in vivo (Badger and Lorimer 1981).

RUBISCO undergoes an apparent activation before it is catalytically active (Lorimer 1981). Activation is usually tested by assaying for activity over a 30 s time period. Thus, non-activated enzyme will have a low activity. This activity is compared to an enzyme which is allowed to incubate in the presence of high Mg^{2+} and high CO_2 before being assayed. The activation process is affected by a number of metabolic effectors. Each of these effectors interacts directly at the catalytic site of RUBISCO. These effectors prevent the binding of the transition analog, carboxyarabinitol 1,5-bisphosphate, to the enzyme (Badger and Lorimer 1981). Positive effectors are the metabolites: NADPH, PGA and fructose 1,6-bisphosphate (FBP) (Hatch and Jensen 1980). Negative effectors are: ribulose 5-phosphate (R5P) and glucose 6-phosphate (G6P) (Hatch and Jensen 1980; Badger and Lorimer 1981). Apparently positive effectors work by stabilising the binding of CO_2 and Mg^{2+} to RUBISCO (Badger and Lorimer 1981). When a positive effector binds to RUBISCO, a conformational change may occur which sterically inhibits the exchange of CO_2 and Mg^{2+} with the environment (Jordan et al. 1983). When a positive effector binds to the enzyme, it renders the enzyme catalytically inactive because RUBP cannot bind (Lorimer 1981). The many varied results concerning which metabolites are positive or negative effectors might be the result of the particular assay used,

as for instance the order of addition of a metabolite versus CO_2 levels (Badger and Lorimer 1981; Jordan et al. 1983). The binding of various effectors does not influence the ratio of carboxylase to oxygenase activity (Chollet and Anderson 1976).

Another enzyme of particular interest in this thesis, is ribulose 5-phosphate kinase (R5P kinase). This enzyme catalyses the addition of phosphate from ATP to R5P, forming RUBP (Bassham 1965). This enzyme is light-activated and is more active at high pH (Laing et al. 1981). Also, when a chloroplast is illuminated, the ATP level increases and the ADP level falls (Kobayashi et al. 1979a). As ADP is a negative effector of R5P kinase, the activity of this enzyme increases (Gardemann et al. 1983). Apparently ADP binds at the ATP site on the protein. Other metabolites, such as PGA, FBP and 6-phosphogluconate affect R5P kinase activity. Specifically, when a chloroplast is illuminated, 6-glucose phosphate dehydrogenase is inactivated (Lendzian and Bassham 1975), thus decreasing 6-phosphogluconate levels in the light. As the later metabolite is a negative effector, the R5P kinase activity increases in the light (Gardemann et al. 1983). Both P_i and FBP are negative effectors of R5P kinase (Gardemann et al. 1983). How R5P kinase is regulated against the complex interaction of these many metabolites in vivo, has never been succinctly discussed in the literature.

FBPase was also examined in this thesis. This enzyme is

affected by Mg^{2+}, pH and sulfhydyrl reagents (Lilley 1983). Evidence from studies of metabolite levels in **Chlorella** showed FBP levels to rise upon illumination, after which they gradually decline. Bassham and Kirk (1968) argued that the above results constituted evidence for light activation of the enzyme. Similarly, Leegood and Walker (1980) have directly measured FBPase activity from intact leaves, by using a rapid protein extraction procedure. They showed that FBPase required about 10 min of illumination in order to reach maximum activity. There exists a direct correlation between the intensity of light (versus darkness) and the extractable FBPase activity. Also, CO_2 concentrations 3 to 14 times that of normal air, decreased the extractable activity (Leegood and Walker 1980). Whether the high CO_2 increased the presence of certain negative effectors, or affected some light-mediated electron transfer process, was not determined.

How the individual activities of the PCR cycle enzymes are coordinated to meet the various demands for PCR cycle carbon in metabolism, needs to be ascertained. Since the level of P_i and phosphorylated metabolites are obviously interrelated to the amount of carbon present in the chloroplast, and to the amount exchanged to the cytoplasm, Heldt et al. (1978) reasoned that the level of P_i may be a controlling factor in the regulation of photosynthetic CO_2 fixation. When intact chloroplasts were incubated at various ratios of P_i to PGA (both are readily transported into the

chloroplast), they found RuBISCO to be inactivated under certain conditions, even when an excess of RuBP was present in the chloroplast. From this they concluded that an optimum P_i to phosphorylated metabolite ratio is necessary for maximum rates of CO_2 fixation. If too much P_i is converted into a phosphorylated metabolite, in order to prevent the complete loss of P_i for other processes (e.g. ATP synthesis), RuBISCO is inactivated. Any unusual disturbance in carbon utilisation might invoke this P_i regulatory mechanism.

A light mediated system exists in chloroplasts that can alter the activities of a number of chloroplast-localised enzymes. Reduced ferredoxin via ferredoxin-thioredoxin reductase donates an electron to thioredoxin (Wolosiuk and Buchanan 1977; Buchanan 1980; Hammel et al. 1983). Thioredoxins are a group of small MW proteins which undergo reversible oxidation and reduction through changes in sulfhydryl groups (S-S to 2SH). Upon illumination, reduced thioredoxins are made which in turn can reduce the sulfhydryl groups on specific proteins thereby activating these proteins. To transfer an electron from PSI to a stromal enzyme is a function of the thioredoxins. There is a greater number of reduced groups in fructose 1,6-bisphosphatase (FBPase) after illumination of chloroplasts, when compared to the number of reduced groups of FBPase after dark treatment (Slovacek and Vaughn 1982). When

noncyclic electron transfer is chemically inhibited, FBPase activity remains low in the light and the number of sulfhydryl groups is unchanged from the dark level (Slovacek and Vaughn 1982). From the work of Anderson (1979) a second system, although not as well characterised, has been suggested.

Photorespiration

Photorespiration is the light-dependent evolution of CO_2 (Krotkov 1963). The rate of photorespiration is dependent upon the ratio of O_2 to CO_2 within the cell. This is in contrast to a second CO_2 evolving mechanism, that is to say that from dark respiration. The rate of dark respiration is independent of the CO_2 concentration above a 2% O_2 level (Canvin 1979). One hypothesis relating photorespiration to the gas exchange properties of the leaf, concerns the dual reaction mechanism of RuBISCO (Laing et al. 1974; Lorimer and Andrews 1981). Kinetic studies have shown that O_2 and CO_2 compete for the catalytic site of RuBISCO (Bowes and Ogren 1972; Laing et al. 1974; Lorimer 1981). The first product of photorespiration is considered to be phosphoglycolate. Lorimer et al. (1973) showed that O_2 was incorporated into the carboxyl group of phosphoglycolate with the aid of isolated RuBISCO. The oxygenation of RuBP by RuBISCO is considered to be the only way to explain plant photorespiratory characteristics (Ogren 1984). The details of the reaction mechanism have yet to be worked out.

Bjorkman (1966) showed that the presence of O_2 in the environment decreases the rate of photosynthesis in plants which synthesize PGA as the first product of CO_2 assimilation. The O_2 inhibition of photosynthesis is manifested by an increased quantum requirement and a lowered growth rate in normal air, as compared to CO_2-enriched or low O_2 containing air (Bjorkman et al. 1969; Quebedeaux and Hardy 1975; Ehleringer and Bjorkman 1977). Later work showed that photosynthesis by plants (C_4) whose initial CO_2 product is a four carbon metabolite, are not inhibited by 21% O_2. (Chollet and Ogren 1975). In general, C_4 plants can concentrate CO_2 to high levels within a leaf and thus overcome photorespiration (Hatch and Osmond 1976).

For C_3 plants, the reduction of photosynthetic CO_2 exchange caused by O_2 is thought to occur by a direct inhibition of CO_2 fixation, as well as by an increased CO_2 evolution from the leaf (Forrester et al. 1966; Tregunna et al. 1966). The direct inhibition by O_2 is evidenced by the observation that as O_2 concentration is increased, the slope of the curve of apparent photosynthesis versus CO_2 level decreases. The increase in CO_2 release was examined by determining the compensation point. The compensation point represents that point in gas exchange when the rate of CO_2 fixation is equal to the rate of CO_2 evolved by the plant. As dark respiration does not vary with O_2 concentration, the fact that the compensation point was increased in the

presence of O_2 and light, was thought to be due to the stimulating effects of O_2 on photorespiration (Krotkov 1963).

To understand the physiological basis of photorespiration, a biochemical formulation was developed to determine the effects of various O_2/CO_2 ratios on cellular metabolism. It has been shown that the percentage of ^{14}C recovered in serine, glycine and glycolate following photosynthetic CO_2 fixation of $^{14}CO_2$ increases as the O_2 concentration in the environment around the plant increases (Whittingham et al. 1963; Tolbert 1971). In fact, after the first few minutes of photosynthesis, up to 50% of the total ^{14}C recovered is found in these metabolites. The carbon atoms originate from the PCR cycle, since ^{14}C was equally distributed to both carbon atoms of the isolated glycolate (Benson and Calvin 1950; Schou et al. 1950; Hess and Tolbert 1966). It has been suggested that because glycine and serine are rapidly labeled during photosynthesis and the C-1 and C-2 atoms are equally labeled with ^{14}C from $^{14}CO_2$, these metabolites are closely associated with glycolate (Hess and Tolbert 1966). In comparison, other amino acids were slowly labeled and initially their carboxyl group was often involved (Hess and Tolbert 1966). Rabson et al. (1962) had shown that when 2-^{14}C-glycolate was fed to leaves, the first recipients of ^{14}C were glycine and serine. In 1970, Kisaki and Tolbert showed that when 1-^{14}C-glycine was fed to leaf discs a considerable release of $^{14}CO_2$ took place. Little

$^{14}CO_2$ was released with 2-^{14}C-glycine. This is consistent with the conversion of 2 glycine molecules to one molecule of serine. Later, this reaction was localised to the mitochondria (Kisaki et al. 1971). As shown in Figure 1, serine or glutamate are needed to aminate glyoxylate. The aminotransferases have been localised to the peroxisomes and purified. They catalyse essentially irreversible reactions (Nakamura and Tolbert 1983). The analysis of $^{14}CO_2$ photosynthetic products in isolated chloroplasts is also consistent with the origin of glycolate carbon from the PCR cycle (Kirk and Heber 1976).

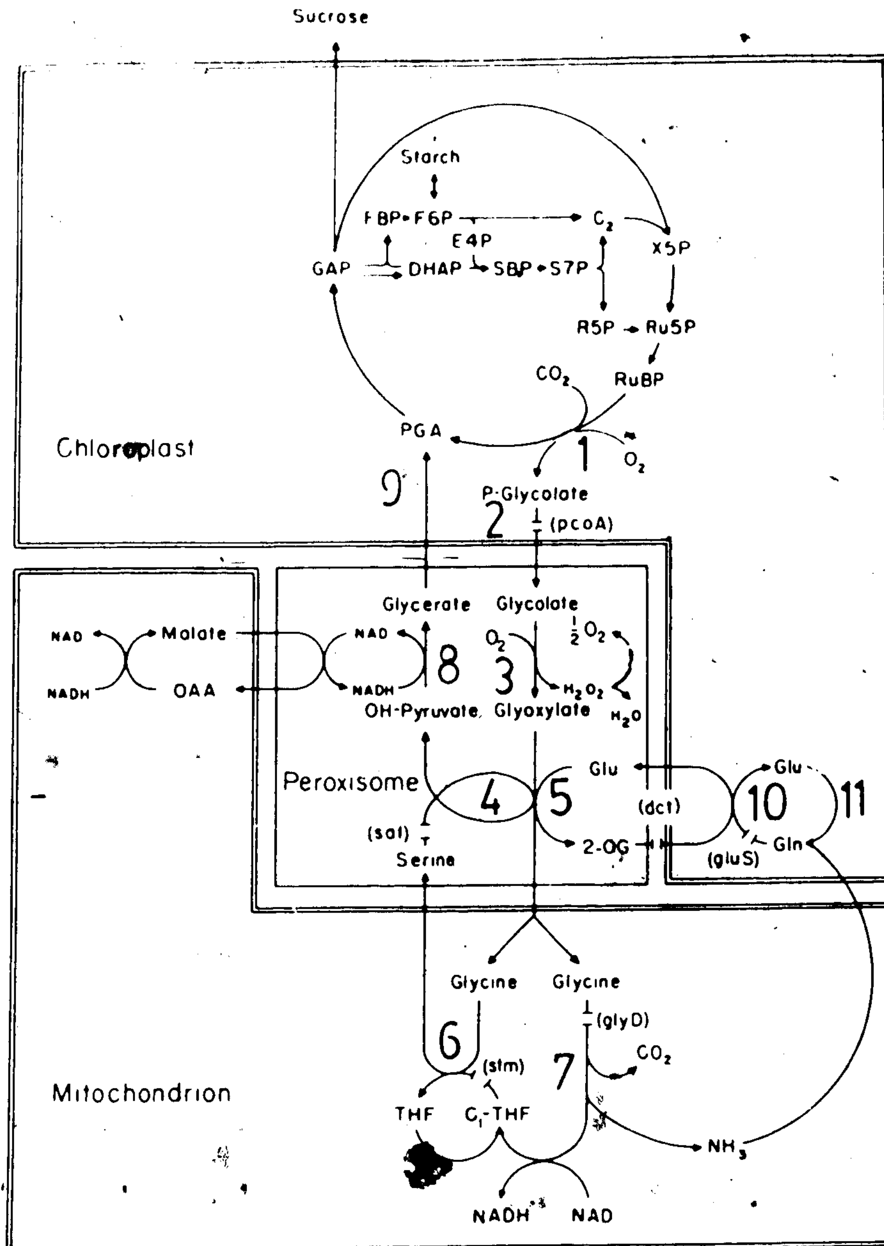
When $^{18}O_2$ is fed to leaves, it is incorporated into the carboxyl oxygens of glycine and serine (Andrews et al. 1971). However, enrichment of the ^{18}O in these groups is less than the source $^{18}O_2$. It is possible that in vivo generating sources (such as the oxidation of H_2O) dilute the $^{18}O_2$. In **Chlorella**, which excretes glycolate, Lorimer et al. (1977) showed at least 90% of the glycolate excreted had ^{18}O incorporated. This, along with the above studies, is consistent with the view that RuBISCO catalyses and is the major route in the synthesis of phosphoglycolate.

Many of the reactions of the PCO cycle have been confirmed by the isolation of mutant plants. In 1979, Somerville and Ogren reported the isolation of an **A. thaliania** plant deficient for phosphoglycolate phosphatase activity and which required a high CO_2 atmospheric content

for growth. The genetic screening procedure used to isolate this mutant was developed from the concept that O_2 and CO_2 are mutually competitive substrates for RuBISCO. Therefore, mutants in the PCO cycle could be isolated missing an enzyme, if their function was not essential for viability. The phosphoglycolate phosphatase mutant (**PCOA**) demonstrated the success of this screen and provided evidence that phosphoglycolate was the precursor to glycolate. When intact plants of this mutant were exposed to $^{14}CO_2$ for 2 min in a photorespiratory atmosphere, 20% of the total label accumulated in phosphoglycolate. Only a small percentage of label was found in glycolate. Other mutants isolated in **A. thaliania** have been found to be deficient for serine-glyoxylate aminotransferase (**SAT**), serine-trans-hydroxymethylase (**STM**), glycine decarboxylase (**GLYD**) and a dicarboxylate transporter protein (**OXOT** or **DCT**). These mutants and their particular characteristics have been recently reviewed (Somerville 1984) and their locations in the PCO cycle is indicated in Figure 1. Using the same screening procedure, Kendall et al. (1983) have isolated a catalase deficient mutant in barley. The isolation of **GLUS** mutants in barley has also been reported (Wallsgrove et al. 1983), a full journal publication has not yet appeared.

The inviability of the photorespiratory-deficient mutants of **A. thaliania** has never been thoroughly examined, but it is assumed to be caused by the decline in net CO_2 fixation (Somerville and Ogren 1983). All the mutants are

Figure 1. A schematic representation of the PCO and PCR cycles. The numbers correspond to the following enzymes: [1] RuBISCO, [2] phosphoglycolate phosphatase, [3] glycolate oxidase, [4] serine-glyoxylate aminotransferase, [5] glutamate-glycolate aminotransferase, [6] serine transhydroxymethylase, [7] glycine decarboxylase, [8] hydroxypyruvate reductase, [9] glycerate kinase, [10] GOGAT, [11] glutamine synthetase. Gene symbols are also given for mutants which have been described. The gene symbols correspond to the enzyme deficiency numbered in the respective reaction step. The only exception is the **OXOT** or also known as **DCT**, which is deficient for a membrane transporter. This scheme is similar to Somerville (1984) and is published with Dr. C.R. Somerville's permission.



Sucrose
Starch
FBP
F6P
C2
E4P
X5P
GAP
DHAP
SBP
S7P
R5P
Ru5P
CO2
RuBP
PGA
Chloroplast
1
O2
9
P-Glycolate
2
(pcoA)
Glycerate
Glycolate
NAD
Malate
NADH
OAA
8
3
O2
½ O2
H2O2
H2O
OH-Pyruvate
Glyoxylate
Peroxisome
4
5
Glu
(dct)
10
11
(sat)
Serine
2-OG
(gluS)
Gln
Glycine
Glycine
(glyD)
6
CO2
(stm)
7
Mitochondrion
THF
C1-THF
NH3
NADH
NAD

characterised by inhibition of photosynthetic CO_2 fixation in normal air. It is believed that this is caused by a block in a PCO cycle reaction, which would prevent carbon return to the PCR cycle and consequently would lead to a lack of PCR cycle carbon. However, in the PCOA mutant, accumulated phosphoglycolate inhibits the PCR cycle directly and inhibits photosynthetic CO_2 fixation (Somerville and Ogren 1979). In addition, the catalase mutant apparently accumulates H_2O_2, which rapidly destroys membranes (Parker and Lea 1983).

If the operation of the PCO cycle is directly related to photorespiration, then the rate of the PCO cycle must be related to the rate of the photorespired CO_2. By feeding $^{14}CO_2$ of a known specific activity to mat[illegible] leaves during steady state photosynthesis, Canvin and colleagues showed the specific activity of glycine and serine approximated the specific activity of the light-dependent and evolved CO_2 (Mahon et al. 1974; Canvin et al. 1976). This is expected if the CO_2 evolved by photorespiration is directly linked to the metabolism of glycine and serine. Furthermore, the rate of carbon flow through glycine and serine was equal to the rate of photorespiration. In 1978, Agrawal and Fock showed that the specific activity of the photorespiratory-evolved CO_2 was nearly identical to that of glycolate.

In summary, the PCO cycle is a complicated pathway, as many reactions are involved and these occur in three

different organelles (Tolbert 1971, 1980; Schnarrenberger and Fock 1976; Lorimer and Andrews 1981; Ogren 1984; Fig. 1). It is thought to have evolved to salvage the phosphoglycolate synthesised as a result of the **RuBISCO** oxygenase activity (Lorimer and Andrews 1981). It is thought that the oxygenase reaction is the result of an evolutionary constraint excercised by the enzyme's carboxylase mechanism (Lorimer and Andrews 1981). The stoichiometry of the pathway dictates that 3/4 of the carbon which flows through the PCO cycle returns to the chloroplast. The remaining 1/4 is released as CO_2 (Lorimer et al. 1977; Lorimer and Andrews 1981).

Nitrogen Metabolism

Nitrogen assimilation also utilises the reduced ferredoxin, ATP and NADPH derived from the photochemical reactions. Most plants acquire nitrogen from the soil as NO_3^-, which is reduced to NO_2^- and then to NH_4^+. The reduction of NO_3^- by nitrate reductase can occur in the root and/or leaf tissue and is localised in the plastids (Beevers and Hageman 1969, 1980; Pate 1973; Oaks and Hirel 1985). The electron donor required by nitrite reductase is reduced ferredoxin (Joy and Hageman 1966). Reduced ferredoxin is also used by fd-dependent GOGAT for the synthesis of glutamate in the leaf (Lea and Miflin 1974). ATP is used by glutamine synthetase for the assimilation of NH_4^+. Experiments with $^{15}NO_3$ have shown that in roots (Yoneyama

and Kumazawa 1974) and in leaves (Bauer et al. 1977) ^{15}N is first incorporated into the amide group of glutamine and then into the amino group of glutamate. It is considered that the principal route for the entrance of NH_4^+ into metabolism is by the glutamine synthetase/GOGAT cycle, since glutamate dehydrogenase (GDH) has a high Km and glutamine synthetase has a low Km for NH_4^+ (Wallsgrove et al. 1983; Oaks and Hirel 1985).

There appear to be two predominate types of GOGAT enzyme in plants, one is NADH-dependent and the other is fd-dependent (Lea and Miflin 1974; Suzuki and Gadal 1984). Both types are found in the leaves and in the roots and are localised to the plastid compartment of the cell. The finding of fd-dependent GOGAT in the root tissue was unexpected, as ferredoxin is a photochemical product and has not previously been isolated from root tissue (Joy and Hageman 1966). Recently, a ferredoxin-like molecule that may be reduced by a NAD(P)H-dependent system has been partially characterised in root tissue (Suzuki et al. 1985). Consequently, a role for fd-dependent GOGAT in root nitrogen assimilation may exist.

The developmental expression of NADH- and fd-dependent GOGAT activities in higher plants is distinct. Generally, fd-dependent GOGAT activity is reduced in etiolated leaf tissue and NADH-dependent GOGAT activity represents the major activity in etiolated tissue (Matoh and Takahashi

1981; Wallsgrove et al. 1982). Upon illumination, the NADH-dependent activity remains constant, but the fd-dependent GOGAT activity increases 10-20 fold. In rice leaves, a similar pattern is observed, except that NADH-dependent GOGAT activity cannot be detected in green leaves (Suzuki et al. 1982). However, in maize leaves, little change in fd-dependent GOGAT activity was detected upon illumination (Harel et al. 1977). How light regulates fd- and NADH-dependent GOGAT gene expression, is unknown. The proteins appear to be immunolgically distinct (Suzuki et al. 1982).

In studies with various tissues, other reductant sources have been used to detect GOGAT activity. NADPH-dependent activity has been observed in cultured tobacco cells and was partially characterised by fd-sepharose chromatography (Suzuki et al. 1984). Whether this is a unique GOGAT protein or represents one of the other GOGAT proteins with the ability to use NADPH as a reductant, is unknown. Matoh et al. (1980) purified pea NADH-dependent GOGAT and showed NADPH could not be used as a reducing source.

The artificial donor, methyl viologen has also been used to detect GOGAT activity. Methyl viologen can be used by the partially purified NADH-dependent GOGAT of C. **reinhardii** (Cullimore and Sims 1981; Marquez et al. 1984) and of pea (Matoh et al. 1980). In fact, the most abundant GOGAT activity in C. **reinhardii** is the one associated with methyl viologen-dependent GOGAT (Marquez et al. 1984). In

contrast, Suzuki et al. 1982, who detected no NAD(P)H-dependent activity in green leaves, reported the existence of methyl viologen-dependent GOGAT in green leaves. The activity of methyl viologen-dependent GOGAT amounted to 50% of the fd-dependent activity. They also reported, that an antibody raised against fd-dependent GOGAT, inhibited methyl viologen-dependent activity, but not NADH-dependent activity.

The magnitude of the photorespiratory nitrogen cycle's influence on leaf metabolism was established with ^{15}N tracer studies and by the use of methionine sulfoximine (MSO). MSO reversibly inactivates glutamine synthetase, a key enzyme involved in glutamine synthesis and NH_4^+ assimilation (Wallsgrove et al. 1983). In a study where [^{15}N,1-^{14}C]-glycine was fed to leaf tissue and also to isolated mitochondria, it was demonstrated that CO_2 and NH_4^+ are released in equimolar amounts (Keys et al. 1978). This release of CO_2 and NH_4^+ is consistent with the sequence of carbon flow through the PCO cycle as proposed by Tolbert (1971) (Fig. 1). Keys et al. (1978) estimated the rate of photorespiratory release of NH_4^+ to be an order of magnitude greater than that of NH_4^+ formed by primary assimilation. Possibly, any block in the enzymatic synthesis of glutamate or glutamine in the cell would be deleterious for growth, as a significant atmospheric loss of NH_4^+ might starve a plant for nitrogen. In another quite similar study, it was shown

that the ^{15}N released from the glycine decarboxylase complex is reassimilated into the amido-N of glutamine (Woo et al. 1982). The remaining ^{15}N from the labeled glycine, was utilised in the synthesis of serine. It was concluded that the GOGAT/glutamine synthetase nitrogen cycle is involved with the recycling of photorespired NH_4^+.

Using a genetic screening procedure to isolate plants deficient in photorespiration, Somerville and Ogren (1980a) isolated recessive, nuclear gene mutants (**GLUS**) deficient in leaf fd-dependent GOGAT activity. These plants were characterised by an accumulation of NH_4^+ only under photorespiratory gas regimes. The authors also observed a concomitant drop in leaf glutamate levels that coincided with a drop in CO_2 fixation. When raised in CO_2 enriched air, the growth and appearance of the **GLUS** and WT plants were indistinguishable. However, when the **GLUS** plants were transferred to normal air, they became yellow in appearance and are inviable in 4 to 6 days.

By their biochemical characterisation of the **GLUS** mutants, Somerville and Ogren (1980a) affirmed the earlier results of Tolbert (1971) and Keys et al. (1978). Firstly, the lack of fd-dependent GOGAT activity prevented sufficient glutamate from being synthesized for the glutamine synthetase reaction. Thus the level of NH_4^+ rose in the leaves of **GLUS** plants photosynthesizing under photorespiratory conditions, whereas NH_4^+ levels remained low in the WT plants under the same conditions. Secondly,

the low level of glutamate apparently reduced the peroxisomal transamination of glyoxylate to glycine as the level of glycine dropped in the leaf. It is axiomatic that such a block in the PCO cycle will reduce PCR cycle intermediates, because PCO cycle carbon re-enters the PCR cycle via the phosphorylation of glycerate to PGA (Tolbert 1971; Fig. 1).

Generally, if plants are exposed to high levels of NH_4^+ through a nutrient solution, changes occur in their morphology and physiology (Givan 1979). Cucumber plants exposed to 14.3 mM NH_4Cl have increased rates of respiration and eventually yellow (Matsumoto et al. 1971). Tomato plants given 10 mM $(NH_4)_2SO_4$ yellow within 3 days and lose thylakoid structure (Puritch and Barker 1967). **A. thaliania** is particularly sensitive to NH_4^+, since as little as 2 mM stunts growth, changes leaf morphology and thickens the leaves (Doddema 1978; Doddema et al. 1979). The specific causes for the above responses is unclear, but a contributing factor might be the inhibition of photosynthesis by uncoupling photophosphorylation (Givan 1979).

When **Chlorella** cells or leaf tissue are exposed to NH_4^+, there is a general increase in the recovery of ^{14}C from $^{14}CO_2$ as recovered in the amino acids, at the expense of sucrose synthesis (Kanazawa et al. 1970; Platt et al. 1977; Kanazawa et al. 1983; Walker et al. 1984). Platt et

al. (1977) incubated alfalfa leaf discs on NH_4Cl and found an increased recovery of ^{14}C in pyruvate, with a decrease in phosphoenolpyruvate (PEP), which was taken as evidence for a stimulated anaplerotic flow of carbon via pyruvate kinase and PEP carboxylase. Pyruvate kinase exists in 2 isozymic forms in spinach leaves and is inhibited by ATP (Baysdorfer and Bassham 1984). Neither isozyme was affected by NH_4^+. The importance of these reactions is emphasized by the fact that the tricarboxylic acid (TCA) cycle intermediates, oxaloacetic acid (OAA) and 2-oxoglutarate, are substrates for the synthesis of aspartate, glutamate and glutamine (Miflin and Lea 1977). The 2-oxoglutarate is strictly generated in the mitochondria, and cannot be synthesized in the chloroplast, due to the lack of the necessary enzymes in the chloroplast. Thus, in the presence of NH_4^+, chloroplast transfered triose-P and PGA are somehow diverted from sucrose synthesis into the TCA cycle. Although models exist regarding the regulation of sucrose synthesis (Stitt et al. 1983; Cseke et al. 1984), none have incorporated the specific effects of NH_4^+ on sucrose synthesis.

Recently, Walker et al. (1984) studied both the effects of NH_4Cl and MSO on phosphorylated carbon metabolism in wheat leaves. In the presence of 30 mM NH_4Cl, amino acid synthesis was increased. In the presence of MSO amino acid synthesis decreased. Because MSO inhibits glutamine synthetase, the essential NH_4^+ assimilation cycle via glutamine synthetase and GOGAT is blocked. In both

treatments, NH_4^+ accumulated and photosynthetic CO_2 exchange was inhibited. However, the level of NH_4^+ was lower in MSO treated plants when photosynthetic CO_2 exchange declined, when compared to NH_4Cl treated plants in which photosynthesis declined. Walker et al. (1984) used the work on the photorespiratory-deficient mutants of **A. thaliania** as supporting evidence for the suggestion that the decline in photosynthetic CO_2 exchange in the MSO treated plants was in part do to a block in the PCO cycle, leading to a depletion of carbon in the PCR cycle. They commented that the ^{14}C content increased in the phosphate ester fraction. Unfortunately, the individual metabolites were not separated and identified. The decline in photosynthesis in NH_4Cl treated plants was thought to be the result of uncoupled photophosphorylation.

The inhibition of photosynthetic CO_2 exchange by MSO thereby preventing the return of carbon through the PCO cycle, is a tenable proposal. However, in maize, a C_4 plant, MSO also inhibits photosynthetic CO_2 exchange (Berger and Fock 1985). C_4 plants have a very typical low rate of carbon flow through the PCO cycle, as evidenced by a low compensation point (Hatch and Osmond 1976; Morot-Gaudry et al. 1980). Berger and Fock (1985) fed MSO to detached leaves of both wheat and maize. In both plants photosynthesis declined and suprisingly, NH_4^+ accumulated. When photosynthesis was 70% inhibited the level of NH_4^+ was

nearly identical in both species. Obviously, there is a great turnover of amino acids in maize. But, more importantly, the data indicate NH_4^+ is inhibitory to leaf photosynthesis and that MSO might inhibit photosynthetic CO_2 exchange by a process independent of carbon flux through the PCO cycle.

Inhibition of Photosynthesis in GLUS Mutants

Somerville and Ogren (1980a) examined the accumulation of NH_4^+ by GLUS plants photosynthesizing in 50% O_2 and 357 ppm CO_2. After 25 minutes of photosynthesis, NH_4^+ was measured at 3 mM in the mutant and 0.4 mM in the WT strain. An NH_4^+ concentration of 3 mM has been reported to inhibit photophosphorylation by thylakoids (Krogmann et al. 1959). It was suggested that NH_4^+ was responsible for the decline in photosynthesis (Somerville and Ogren 1980a).

The putative mechanism for photosynthetic uncoupling by NH_4^+ is based on the fact that H^+ ions are carried across the thylakoid membrane during photosynthetic electron transport (Portis and McCarty 1976). Thus, due to the insular structure of the membrane, a H^+ gradient develops across this membrane. The gradient is normally released through the photophosphorylation complex, producing ATP (Ort and Melandri 1980). In the presence of membrane permeable NH_3 (Crofts 1967), the internal H^+ of the thylakoids react to form NH_4^+. As a result, NH_4^+ is trapped within the thylakoids, causing osmotic swelling (Good 1977).

Consequently, ATP synthesis declines but electron transport may increase in rate as the chloroplasts are now uncoupled from feedback control from the H^+. Therefore, stromal levels for ATP should decrease. A drop in ATP levels, will reduce the level of PCR cycle intermediates, as the normal functioning of this cycle requires ATP for triose-P and RuBP synthesis (Bassham 1965). Further, a lack of RuBP, the immediate substrate for CO_2 fixation, should cause a decline in the rate of CO_2 fixati[illegible]

Although NH_4^+ ca[illegible]uple photophosphorylation with isolated thylakoids, [illegible]uation appears more intricate with intact chloroplasts. Tillberg et al. (1977) showed that 3 mM NH_4Cl reduced the stromal ATP/ADP ratio of spinach chloroplasts by 50%. However, in 1979, the same laboratory showed that ATP levels were not decreased in the presence of 5 mM NH_4Cl (Kobayashi et al. 1979b). It was concluded that NH_4Cl was ineffective as an uncoupler of photosynthesis. Levels of 10 mM NH_4Cl did cause the uncoupling of electron transport in chloroplasts, using either NO_2^- or oxaloacetate as a photosynthetic substrate. Heber and colleagues have also shown that 3 mM NH_4Cl will not inhibit CO_2-dependent photosynthesis (Heber 1984). This would seem to contradict the view that a H^+ gradient is required for ATP synthesis. Heber (1984) did show that this gradient was reduced in the presence of NH_3, but not enough to inhibit ATP synthesis. Work by Givan (1975) with pea chloroplasts, showed 7 mM

NH_4Cl reduced the incorporation of ^{14}C from U-^{14}C-glutamate by 70% into glutamine, via the glutamine synthetase reaction, which requires ATP. As pea chloroplasts isolated from young leaves can transport ATP at high rates, the addition of 2 mM ATP, in the presence of NH_4Cl, restored the incorporation rate to 75% of the control value. As yet no one has reported if NH_4^+ when added to leaves reduces stromal ATP levels. Therefore, it seems unlikely that NH_4^+ accumulation is responsible for the decline in GLUS CO_2 fixation, when values less than 3 mM NH_4^+ were measured. However, Slovacek and Hind (1980) have found that 3mM NH_4^+ levels reduce CO_2 fixation with intact spinach chloroplasts. Therefore, the response of any photosynthetic apparatus to uncoupling may be influenced by growth conditions (and possibly genotypic differences). Consequently, NH_4^+ uncoupling and inhibition of ATP synthesis may actually be the cause for the decline in GLUS photosynthesis.

The isolation of other mutants deficient in a reaction step of the PCO cycle, has led to a reconsideration of what causes the initial decline in photosynthetic CO_2 exchange in the GLUS mutant (Somerville and Ogren 1983; Somerville 1984). The DCT mutant of **A. thaliania**, which is deficient in its ability to mediate the transfer of 2-oxoglutarate and glutamate between the chloroplast and cytosol is the mutant of immediate interest to the present author (Somerville and Ogren 1983). Photosynthetic CO_2 exchange is inhibited in 49% O_2 and 557 ppm CO_2, but unaffected in non-photorespiratory

conditions. There is a drop in the percent recovery of ^{14}C from $^{14}CO_2$ into the PCO cycle intermediates, glycine and serine, and leaf amino acid levels of glycine, serine and glutamate decrease under photorespiratory conditions. There is an increase in NH_4^+ content in the mutant, that is not observed in the WT strain. These characteristics are similar to those reported previously for the **GLUS** mutant (Somerville and Ogren 1980a). However, in the **DCT** mutant there is no apparent increase of NH_4^+ when compared with the WT strain at a time when photosynthetic CO_2 exchange initially declined. The authors suggested that the most likely explanation for the decline of photosynthetic CO_2 exchange, is the depletion of the PCR cycle, as a result of the interconnected carbon pools of the PCO and PCR cycles and the proposed block at the amination of glyoxylate because of the lack of glutamate. This is, apparently, only a tentative reason, as it was noted that the percent recovery of ^{14}C from $^{14}CO_2$ was higher in the fraction containing the PCR cycle intermediates in the **DCT** mutant than in the WT strain. The individual metabolites of the fraction were not directly measured for ^{14}C content.

Other workers have also proposed that any significant loss of carbon into the PCO cycle, without its return, might deplete the PCR cycle of intermediates. Servaites and Ogren (1977) isolated mesophyll cells from soybean and incubated these cells and measured CO_2 fixation rates in the presence

of two inhibitors purported to be PCO cycle specific. In the presence of these compounds, CO_2 fixation is inhibited. The authors suggested that the depletion of PCR cycle intermediates constitited a likely explanation for the reduced photosynthetic CO_2 exchange rates. Unfortunately, no supportive data concerning this point have been presented, and no supportive evidence other than that present in the literature has been quoted.

Kirk and Heber (1976) investigated the effects of a continuous export of carbon away from intact chloroplast during photosynthesis. They specifically measured the rate of synthesis of glycolate by chloroplasts, photosynthesizing under photorespiratory gas regimes. In an isolated intact chloroplast experiment, glycolate was exported and does not return to the chloroplast. Therefore, this study is analagous to other studies in which the PCO cycle is either chemically or genetically blocked. Kirk and Heber (1976) found that increased glycolate synthesis was accompanied by a depletion of PCR cycle intermediates, and a decrease in CO_2 fixation. In fact, if the rate of glycolate synthesis was too great, CO_2 fixation ceased.

A third explanation for the decline in photosynthesis has been offered from the work of Creach and Stewart (1982). Aminoacetonitrile (AAN), an analog of glycine, was fed to soybean leaves in order to inhibit the glycine decarboxylase reaction. Photosynthesis was not inhibited at a concentration level of 2% O_2. At 21% O_2 photosynthesis was

inhibited resulting in an increase in total glycine, and glyoxylate. The authors reported that the total level of RuBP increased relative to a control plant (no AAN). They suggested that a block in the PCO cycle in one way or another deactivates RuBISCO.

This proposal has recently been supported by the finding that RuBISCO was inactivated during the course of photosynthesis under photorespiratory gas regimes in a number of the **A. thaliania** PCO cycle mutants with the exception was the **PCOA** mutant (Chastain and Ogren 1985). Clearly, Chastian and Ogren (1985) provided evidence for an association between the decline in RuBISCO activity and the inhibition of photosynthetic CO_2 exchange in a photorespiratory atmosphere for the **STM**, **SAT** and the **STM** mutants. The **DCT** and **GLUS** mutants required twice the time for inhibition to set in. However, close scrutiny of the data presented for the first group of mutants listed above, shows that the inactivation of RuBISCO appeared to lag by 1 to 2 min behind that of the decline in photosynthesis. Whether or not the inactivation of RuBISCO has a causative role in the process which leads to the decline in photosynthetic CO_2 exchange, is uncertain. No explanation was offered with regard to possible mechanisms responsible for the decline in RuBISCO.

Finally, Cook and Tolbert (1982) reported that glyoxylate can inhibit isolated RuBISCO. Recently, Cook et

al. (1985) reported that glyoxylate may form a Schiff base with lysine which is required for CO_2 activation of RuBISCO. The formation of such a Schiff base would inactivate the enzyme. The Ki observed amounted to 3.3 mM. In the work of Creach and Stewart (1982) an increase in glyoxylate levels was reported when the PCO cycle was inhibited by AAN. Somerville and Ogren (1981) also observed an increase in glyoxylate levels with the STM mutant. Since the glycolate transporter can also transfer glyoxylate (Howitz and McCarty 1985), there exists a possibility that if glyoxylate accumulates in the GLUS mutant, RuBISCO would be inhibited. Glyoxylate can be metabolised in isolated peroxisomes to CO_2 and formate (Grodzinski 1979), or be oxidised by H_2O_2 (Grodzinski 1978). Not withstanding this, the rate of glyoxylate decarboxylation is unaffected in catalase-deficient mutants of barley (Kendall et al. 1983). It is not known whether or not glyoxylate accumulates to significant levels and is transported into the chloroplasts under in vivo conditions.

Materials and Methods

Plant Material

Strains. **Arabidopsis thaliania** (L.) Heyn., race Columbia (Redei 1970) and several derivative lines were used throughout this study. The mutant strain, CS 254, was recovered in a screen for photorespiratory mutants and requires high CO_2 for normal growth (Somerville and Ogren 1980a). Except for the **GL-1 A. thaliania** strain, all other **GLUS** strains used in this study were isolated by Dr. C. S. Somerville, as described by Somerville and Ogren (1982a). The **GL-1** strain was provided by Dr. M. Koorneef (Dept. of Genetics, Agriculture U., Wageningen, The Netherlands). The strain is described by Koorneef et al. (1982) and is lacking epidermal trichomes.

B. napus ("Altex") seeds were obtained from Mr. Ducan Campbell (Dept. of Plant Sci., Univ. of Alberta).

Plant growth conditions. **A. thaliania** was grown from seed in a perlite-sphagnum-vermiculite (1 : 1 : 1) mixture in controlled environment chambers, using continuous light (200 to 335 $\mu E\ m^{-2}\ s^{-1}$, PAR). The light intensity varied between the different growth chambers, however, all plants used for any one set of experiments originated from the same chamber (unless otherwise noted in the text). Light was provided by a mixture of cool white fluorescent and incandescent bulbs. The temperature was maintained at 20 to

22 °C and the relative humidity was between 50 to 70%. Seeds were sown by mixing them in 0.15% agar after which the mix was pipetted into 12.5 cm pots. The planting density was approximately 0.5 plants cm^{-2}. The pots were covered with polyethylene wrap for 2 weeks, in order to maintain a high humidity content. After 5-6 days, perforations were cut in the wrap, to allow watering and gas exchange. Plants were given a 12 to 16 hr dark period every 5 to 6 days, to reduce starch levels. Plants were watered every other day and nutrients were given once a week. The macronutrient solution was (ml l^{-1}): 1.0 M KNO_3 (5), 1.0 M $MgSO_4$ (2.0), 1.0 M $Ca(NO_3)_2$ (2.0), 1.0 M KH_2PO_4 (pH 5.8; 2.5), 2.4% (w/v) Sequesterene 330 Fe (2.0). After dilution of the macronutrient solutions with water to 1 l, 1 ml of micronutrient solution was added, which contained: 70 mM H_3BO_3, 14 mM $MnCl_2$, 10 mM NaCl, 1 mM $ZnSO_4$, 0.2 mM $NaMoO_4$ and 0.01 mM $CaCl_2$ (Somerville and Ogren 1982b).

For the assay of enzymes from root tissue, the plants were grown in 500-ml plastic containers, containing sterilised nutrient solution. The seeds were first sterilised by washing twice in 20% hypochlorite (plus 2 drops Tween-20 100 ml^{-1}), followed by two washes in 96% ethanol and three final rinses with sterile water (Dr. Roberta Smith, Dept. of Plant Sciences, Texas A&M Univ., per. comm.). The seeds were initially germinated in petri dishes in half-strength nutrient solution solidified with

0.75% (w/v) Bacto agar (Doddema 1978) before transfer to the hydroponic containers.

For most experiments the **A. thaliania** plant used had reached a mature rosette stage of plant development (3.5 to 5 weeks).

B. napus plants were grown in a 12 hr / 12 hr day-night cycle, with a temperature of 24 °C / 18 °C. Plants were used after 10 to 40 days of growth. The light intensity during growth was 250 µE m^{-2} s^{-1}. The same nutrient solution as described for **A. thaliania** was used. The plants were grown either in vermiculite or hydroponically in 4 quart plastic containers. In order to obtain root tissue hydroponically grown plants were cultured. Before mixing into the final solution the separate hydroponic solutions were sterilised by autoclaving. For hydroponic experiments seeds were surface sterilised in a solution containing water : H_2O_2 : (96%) ethanol (10 : 10 : 1) after which they were rinsed in sterile water (Mr. D. Campbell, pers. comm.).

Genetic crosses. A. thaliania is normally a self-pollinating species (Redei 1970). However, for genetic studies, plants were crossed by hand pollinating emasculated plants with pollen from another source. Only one genetic marker was used in complementation crosses. Plants to be used as the maternal line were crossed into a glaborous strain, GL-1. The F_1 of the various **GLUS** crosses were tested for high CO_2 growth requirement and for enzyme activity.

Mutagenesis

Mutagenesis protocol. The following procedure was based on Meinke and Sussex (1978). Mature seeds of CS 37, CS 66 and CS 254 were soaked for 10 min in water and rinsed. Ethyl methane sulfonate (EMS) (Sigma Chemical Co.) was added (0.25% vol/vol) and the seeds remained in the above solution for 12 hr after which they were washed in water for 12 hr. The seeds were sown in 40 x 30 cm plastic flats, containing vermiculite, and raised in a CO_2-enriched (0.6-1.0% CO_2) growth chamber. Mature seeds (M_2) were collected and again planted in flats as described above. Phenotypic revertants were studied physiologically only after a further 3 generations of genetic selfing.

Imposition of Photorespiratory Stress.

Growth protocol. All plants to be studied for the effects of photorespiration on growth and physiology were first raised in a chamber containing approximately 1% CO_2 (the remaining air). This was to ensure a uniform growth and the procedure aided the selection of vigorous plants for further studies. After 3-4 weeks the plants were removed to a chamber containing normal air (21% O_2 and approximately 340 ppm CO_2). Only plants at the same developmental stage and of the same size were used. For gas exchange analysis, plants were removed directly from the growth chamber and allowed to equilibrate in the photosynthetic chamber in the

dark for 30 min before the experiment was begun.

Enzyme Assays

Assay of fd-dependent GOGAT activity. Generally, 50 to 200 ul of a crude extract was used per 1 ml of assay. Crude extracts were prepared by grinding 200 to 400 mg fw of tissue in 2.0 to 3.0 ml of a buffer. For leaf tissue, only mature leaves were used. For root tissue, the apical region (3 to 6 mm) was used. The grind buffer used for grinding in GOGAT (EC 1.4.7.1) assays was: 50 mM K phosphate (pH 7.6), 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol and 0.05% Triton X-100. The homogenate was centrifuged at 30,000 g for 40 min, the supernatant was dialysed for 4 to 6 hr against 50 mM K phosphate (pH 7.6), 0.5 mM EDTA, 0.5 mM EGTA and 5 mM 2-mercaptoethanol. In the kinetic studies with purified GOGAT enzyme, approximately 20 to 50 µl of enzyme was used per 1 ml. In a final volume of 1.0 ml the assay mixture contained 50 mM HEPES (pH 7.6), 1.0 mM 2-^{14}C-oxoglutarate (0.5 µC_i / µmol) (Amersham), 2.5 mM glutamine, 5.0 mM aminooxyacetate, 50 µM EDTA, 50 µM EGTA, 2.5 mM 2-mercaptoethanol, 75 µg ferredoxin, 0.8 mg $Na_2S_2O_4$, and 0.8 mg $NaHCO_3$ (Somerville and Ogren 1980a). The dithionite solution was made just prior to use. 2-oxoglutarate was prepared the day of the experiment and in 20 mM NaH_2PO_4 (pH 7.4). The reaction was initiated with dithionite and terminated after 10-15 min at 20 °C by the addition of 30 ul

of 3% H_2O_2 and vortexing to oxidise the dithionite. To separate the 2-oxoglutarate from glutamate the reaction was applied to a 0.5 x 3 cm (in a glass pasteur pipette) Dowex-50 (H^+) column (Sigma Chemical Co.) (Somerville and Ogrén 1980a). The assay vial was rinsed with 3 ml water and the rinse water was added to the column. The radioactive glutamate was eluted with 3 ml of 4 M HCl and the ^{14}C detected by liquid scintillation spectroscopy.

Methyl viologen-dependent GOGAT activity. This assay was performed as described above except that per assay instead of ferredoxin 100 µg of methyl viologen was substituted.

NAD(P)H-dependent GOGAT activity. In a final volume of 1.0 ml, the assay mixture contained 50 mM HEPES (pH 7.6), 2.5 mM 2-^{14}C-oxoglutarate (0.5 μC_i / µmol), 2.5 mM glutamate, 7.5 mM aminooxyacetate, 50 uM EDTA, 50 uM EGTA, 2.5 mM 2-mercaptoethanol and either 0.8 mM NADH or NADPH. Both NADH and NADPH were prepared the day of the experiment in 50 mM K phosphate (KH_2PO_4/Na_2HPO_4) (pH 7.5). The assay was terminated as above and products detected as above.

Assay for RuBISCO activity. Plants were killed by immersion in liquid N_2. Mature leaves were removed under liquid N_2 and were then ground at 4 $^{\circ}C$ in a Ten-Broeck homogeniser, containing an ice-slurry of RuBISCO-buffer: 50 mM Tris-Cl (pH 8.1), 5 mM DTT, 0.25 mM EDTA and 0.01% NP-40.

Approximately 100 to 250 mg fw leaf tissue was ground in 2.5 ml of RuBISCO-buffer. The homogenate was squeezed through a layer of Miracloth (Calbiochem) and immediately assayed for RuBISCO (EC 4.1.1.39) activity at 22-24 ^{o}C, in serum-capped mini-vials. The assay mixture (final volume 700 µl) consisted of 50 to 100 µl of extract added to the following RuBISCO assay-buffer: 50 mM Bicine (CO_2 free) (pH 8.3), 5 mM DTT, 8.5 mM $MgCl_2$, 0.1 mM Na_2·EDTA, 0.6 mM RuBP and 12.0 mM $NaH^{14}CO_3$ (1.0-2.5 μC_i/ µmole) (Amersham and New England Nuclear) (Lorimer et al. 1976). The CO_2-free solution was prepared by first purging the solution with N_2 at pH 5.0 before adjusting the pH to 8.3. The solution was made the day of use and kept within a serum-capped vial. The min-ivials were previously flushed with N_2 before adding the $NaH^{14}CO_3$. The initial RuBISCO activity was determined during a 45 s incubation period before stopping the reaction with 0.5 ml of 4 M formic acid. Formic acid-terminated reaction vials were then flushed with N_2 gas to dryness and the amount of acid-stable ^{14}C radioactivity resuspended in 250 µl of 50 mM formic acid and scintillation fluor added (Anderson and McClure 1973). Radioactivity was determined by scintillation spectrometry. Fully activated RuBISCO activity was determined in the same way except that the extract was incubated in the assay mix without the $NaH^{14}CO_3$ (but in the presence of 8.5 mM $NaHCO_3$) and RuBP at 20 ^{o}C for 15 min prior to assay.

Assay for fructose bisphosphatase activity. The procedure used was basically the method described by Heber et al. (1982) which measured the reduction of NADP (Sigma Chemical Co.). Whole plants were killed by immersion into liquid N_2 after the appropriate time interval. Mature leaves were ground in a Ten-Broeck homogeniser containing an ice-slurry of FBP-grinding buffer: 50 mM Tris-Cl (pH 7.8), 5 mM $MgCl_2$, 1 mM EDTA and 0.05% Triton X-100. The sample was then squeezed through Miracloth and assayed at 20 °C. Aliquots of 50 to 250 µl were added to 750 to 950 µl of FBP-assay buffer: 20 mM triethanolamine-Cl (pH 7.9), 5 mM $MgCl_2$, 0.2 mM EDTA, 1.0 mM FBP, 0.2 mM NADP, 10 units glucose-6-phosphate isomerase (Sigma Chemical Co.) and 1 unit glucose-6-phosphate dehydrogenase (Sigma Chemical Co.). This enzymes identification is EC 3.1.1.11.

Assay for ribulose 5-phosphate kinase activity. This procedure follows Laing et al. (1981). Leaf tissue was killed and ground as for FBPase. An aliquot (75 to 300 µl) of the homogenate was added to 575 to 800 µl of R5P-assay buffer: 50 mM Tris-Cl (pH 7.8), 5 mM $MgCl_2$, 0.5 mM R5P, 2.0 mM ATP and 8.5 mM $NaH^{14}CO_3$ (1 µCi/ µmole) at 20 to 22 °C. The reaction was stopped after 45 s by adding 55-µl of 2 M HCl. Subsequently, the assay was neutralised with NaOH containing 4 mM dichlorophenolindophenol (DCPIP) and RuBP levels were determined. The DCPIP was present as an oxidant to inhibit R5P kinase (EC 2.7.1.19) activity (Sicher et al. 1979).

Aliquots of 400 to 900 µl were added to the RuBISCO assay buffer, containing 50 µg of purified RuBISCO from **B. napus**, in a final volume of 1.0 to 1.5 ml with 8.5 mM $NaH^{14}CO_3$ (2 µCi/ µmole). The assay was acid-terminated 30 min later, dried under N_2 gas and resuspended in formic acid. The radioactivity was determined by liquid scintillation spectroscopy.

Assay for NAD-malic enzyme. NAD-malic enzyme (NAD-ME) (EC 1.1.1.39) was assayed according to Sayre et al. (1979). Leaves (400 mg fw) were ground in 2.5 ml of ME-buffer: 50 mM HEPES (pH 7.6), 5.0 mM $MnCl_2$, 10 mM 2-mercaptoethanol, 0.25 mM EDTA and 1% (w/vol) BSA. The homogenate was filtered through 4 layers of cheesecloth and Triton X-100 was added to give 0.5% (w/vol) and centrifuged 45 s in an Eppendorf microcentrifuge. The supernatant was passed through a Sephadex G-25 (Pharmacia Fine Chemicals) column and the void volume collected and kept on ice under N_2 gas until 15 min before assaying. The enzyme was assayed by following the formation of NADH at 340 nm, at 20 °C. The assay mix was 25 mM HEPES-KOH (pH 7.4), 5 mM malate, 2.5 mM NAD, 5.0 mM $MnCl_2$, 0.15 mM CoA, 5 mM DTT and 0.4 mM EDTA.

Assay for NADH-glutamate dehydrogenase. For the NADH-glutamate dehydrogenase (NADH-GDH) (EC 1.4.1.2) assays, leaves (200 mg fw) were ground in 5 ml of GDH-buffer: 50 mM Tris-Cl (pH 7.8), 1 mM $CaCl_2$ and 0.1% (w/vol) Triton X-100. The homogenate was centrifuged at 10,000 g for 20 min, and

the resulting supernatant was passed through a Sephadex G-25 column. The void volume was collected from the Sephadex G-25 and assayed according to Miflin (1974). GDH was assayed by the decrease in NADH absorbancy at 340 nm. The assay mix was 50 mM Tricine-KOH (pH 7.2), 1 mM $CaCl_2$, 0.2 mM NADH, 10 mM NH_4SO_4 2mM aminooxyacetic acid and initiated with 10 mM 2-oxoglutarate (prepared in 20 mM $NaPO_4$, pH 7.4).

Assay for phosphoenolpyruvate carboxylase. The PEP carboxylase (EC 4.1.1.31) (or PEPcase in the tables) activity was measured similarly to that for RuBISCO. Leaves were ground in the RuBISCO-buffer and assayed at 20°C by measuring $^{14}CO_2$ incorporation into acid-stable products. The assay mix contained in a final volume of 1.0 ml: 50 mM Tris-Cl (pH 8.5), 5 mM $MgCl_2$, 5.0 mM PEP, 4.0 mM 2-mercaptoethanol, 5 mM Na-glutamate and 10 mM $KH^{14}CO_3$ (0.1 µCi / µmol). The reaction was initiated by the addition of the enzyme (100 to 250 µl of extract) and terminated after 3.0 min with 0.2 ml of 4 M formic acid. The assay vials were dried at 80°C (Quebedeaux and Chollet 1975). The acid-stable ^{14}C was determined by liquid scintillation spectroscopy.

Assay for catalase. For the determination of catalase (EC 1.11.1.6), approximately 100 mg of leaves were homogenised in 3 ml of CAT-buffer: 100 mM K phosphate (pH 7.0). The extract was centrifuged for 45 s in a microcentrifuge and the void volume collected from a

Sephadex G-50 column. The enzyme was assayed in 2 ml of 50 mM K phosphate buffer (pH 7.0) containing 0.[illegible]5% Triton X-100 and the reaction was initiated by the a[illegible]on of 32 µl 1.8 mM H_2O_2 (prepared in 50 mM KH_2PO_4). The hydrolysis of H_2O_2 was measured by the reduction of absorbance at 240 nm (Luck 1965).

Assay for cytochrome c oxidase. Cytochrome c oxidase (EC 1.1.3.1) was assayed similarly to Hackett (1964), with 20 to 200 µl of extract pipetted into the bottom of a 1.0 ml cuvette, to which 20 µl of 4.0% digitonin was added, mixed and allowed to sit for 45 to 60 s. Then 500 to 600 µl of 0.1 M K phosphate (pH 7.2) and 150 µl of 1.5 mM cytochrome c reduced with dithionite was added and mixed. The absorbance at 550 nm was followed.

Partial Purification of Glutamate Dehydrogenase

Homogenisation and chromatography. The isolation procedure employed the assay buffer as described by Miflin (1974). Approximately 25 grams of frozen leaves were ground in separate batches in a large Ten-Broeck, containing 50 mM Tris-Cl (pH 8.0), 1.0 mM EDTA, 5 mM 2-mercaptoethanol and 0.02% Triton X-100. The homogenate was squeezed through 6 layers of cheesecloth and then centrifuged at 20,000 g for 20 min. A 30 to 50% $(NH_4)_2SO_4$ precipitate was taken at 4 °C. The pellet was resuspended in 50 mM Tris-Cl (pH 8.0), 0.5 mM EDTA and 2.5 mM 2-mercaptoethanol and dialysed

overnight in the same buffer. The protein was then applied to a 1.5 x 30 cm Ultragel ACA-22 (Pharmacia Fine Chemicals) column. The column was equilibrated in 50 mM Tris-Cl (pH 7.8), 0.5 mM EDTA and 2.5 mM 2-mercaptoethanol. Peak activity fractions eluting from the column were pooled and assayed for the determination of the Km of NH_4^+.

Isolation of GOGAT Proteins

Purification of Root NADH-dependent GOGAT.

Homogenisation and $(NH_4)_2SO_4$ precipitation. Approximately 250 grams of frozen root tissue was blended in 1 l of an ice-slurry of 25 mM K phosphate (pH 7.4), 12.5 mM 2-mercaptoethanol and 5 mM EDTA (Wallsgrove et al. 1977). After blending, Triton X-100 was added to 0.05%. The homogenate was squeezed through 8 layers of cheesecloth and centrifuged at 5000 g for 20 minutes. The supernatant was then clarified by filtration through glass wool. This filtrate was fractionated between a 30-55% $(NH_4)_2SO_4$ (enzyme grade; Sigma Chemical Co.) concentration. The pellet was gently resuspended in a minimum volume of 20 mM K phosphate, 5 mM 2-mercaptoethanol, and 2.0 mM EDTA. The K phosphate was prepared by mixing 2.72 K_2HPO_4 and 0.54 g of KH_2PO_4 into 1 L of water. After overnight dialysis against the DEAE column buffer (20 mM K phosphate, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, pH 7.4), the suspension was not completely soluble and was therefore centrifuged at 2000 g for 15 min and the

pellet discarded.

DEAE chromatography. The DEAE cellulose (Sigma Chemical Co.) was prepared by adding approximately 200 g to 500 ml of 95% ethanol (Clark and Switzer 1977). The suspension was stirred followed by filtration on a Buchner funnel. The precipitate was resuspended in 1 l water, filtered again and then resuspended in 1.0 M NaOH and filtered as above. The precipitate was resuspended 3 times in water and filtered each time. It was finally resuspended in the appropriate column buffer and allowed to settle and the fines decanted. DEAE cellulose was stored at 4 °C in DEAE column buffer. The DEAE was poured into a column and equilibrated with DEAE column buffer by running 10 times the bed volume through an approximate 5.0 x 35 cm column. The dialysed protein solution was layered onto the column after being diluted to less then 5 mg ml^{-1}. The NADH-dependent GOGAT activity was eluted with a linear gradient of 0.0 - 0.3 M KCl in DEAE column buffer. The peak fraction was concentrated by 60% $(NH_4)_2SO_4$ precipitation, and dialysed overnight in the DEAE column buffer.

ACA-34 chromatography. The dialysed protein solution was layered on 2 x 45 cm column of ACA-34 (Pharmacia Fine Chemicals) and eluted with 20 mM K phosphate (pH 7.4), 5 mM 2-mercaptoethanol and 0.5 mM EDTA. The peak NADH-dependent GOGAT activity fractions were concentrated by layering sucrose crystals on a dialysis bag containing the protein.

The enzyme was dialysed overnight against storage buffer containing 20 mM K phosphate, 5 mM DTT, 0.1 mM EDTA and 20% glycerol.

Purification of leaf fd-dependent GOGAT. Leaves (390-480 grams) without stems were washed in cold tap water and homogenised in 2 l 100 mM K phosphate (pH 7.5), 5 mM EDTA and 12.5 mM 2-mercaptoethanol (Wallsgrove et al. 1977). A total of 16.5 grams of enzyme-grade $(NH_4)_2SO_4$ per 100 ml of extract was added slowly. After the solution was dissolved, the homogenate was kept at 4 ^oC for 90 min, without stirring. The homogenate was centrifuged at 10,000 g for 30 min. A total of 14.5 grams of $(NH_4)_2SO_4$ was then added per 100 ml and the solution allowed to sit after complete dissolution. Subsequently the solution was centrifuged and the pellet resuspended in a minimum volume of 20 mM K phosphate (pH 7.4), 2.0 mM EDTA and 10 mM 2-mercaptoethanol. The suspension was dialysed overnight and then centrifuged at 2000 g to remove insolubles.

DEAE chromatography. The homogenate was applied to an approximately 5 x 45 cm column of DEAE cellulose, which had been equilibrated with 20 mM K phosphate (pH 7.4), 0.5 mM EDTA and 10 mM 2-mercaptoethanol. The homogenate was applied at a rate of approximately 200 ml hr^{-1} (Clark and Switzer 1977). The column was washed with approximately 500 ml of DEAE column buffer. The activity was eluted with a linear

gradient of 0-0.3 M KCl in DEAE column buffer. Pooled active fractions were $(NH_4)_2SO_4$ precipitated, resuspended in 20 mM K phosphate (pH 7.4), 0.1 mM EDTA and 10 mM 2-mercaptoethanol and dialysed overnight. On occasion, it was necessary to run a smaller DEAE column (2 x 30 cm), as the peaks were quite large at times.

ACA-34 chromatography. The extract was layered onto a 2 x 45 cm column at a rate of 20 ml hr^{-1}. The buffer used was 20 mM K phosphate (pH 7.4), 7.5 mM 2-mercaptoethanol and 0.1 mM EDTA. Three-ml fractions were collected. For maximum purity, only the fractions within the Gaussian portion of the activity peak was pooled. The pooled fractions were concentrated against sucrose and dialysed overnight against 20 mM K phosphate (pH 7.4), 0.1 mM EDTA, 7.5 mM 2-mercaptoethanol and 10% glycerol.

Hydroxylapatite chromatography. A column of approximately 2 x 12.5 cm was equilibrated with 10 mM K phosphate, 0.1 mM EDTA and 5 mM 2-mercaptoethanol (C. S. Somerville, pers. comm.). Activity was eluted in a linear gradient of 80 - 400 mM K phosphate. The peak fractions were concentrated against sucrose and dialysed in 20 mM K phosphate (pH 7.4), 0.1 mM EDTA and 10 mM DTT and 10 % glycerol. Purified protein was stored at 4 ^{o}C.

Sucrose gradient centrifugation. A sucrose gradient was performed according to Martin and Ames ([illegible]). A sample of

the final protein preparation was dialysed overnight in 20 mM K phosphate (pH 7.4), 1 mM Na_2EDTA, 2.5 mM DTT and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co.). A 0.1 ml sample was laid onto a 5 - 20% sucrose (Ultrapure, Sigma Chemical Co.) gradient in a similar buffer, without PMSF, and centrifuged in a SW 50.1 rotor for 16 hr at 150,000 g and at 2-3 $^{\circ}C$. Individual tubes were punctured and 200 ul fractions were collected. GOGAT activity was assayed enzymatically. BSA (fatty-acid free, Sigma Chemical Co.), yeast alcohol dehydrogenase (Boehringer Mannheim) and crystalline human hemoglobin (Calbiochem) were used as MW standards, and assayed for their presence at 280 nm. Approximately 0.5-1.2 mg of the standard protein was used in each tube.

Differential centrifugation. The location of GOGAT activities within the cell was determined by differential centrifugation of tissue extracts. Approximately 2 grams fw of leaf tissue was chilled to 4 $^{\circ}C$ and then suspended in 4 ml of grinding buffer. The grinding medium was 75 mM HEPES (pH 7.6), 0.4 m sucrose, 7.5 mM mercaptoethanol, 1 mM $MgCl_2$, 0.2 mM EDTA and 0.5% BSA. The tissue was ground gently in a loose fitting Ten-Broeck homogeniser. The homogenate was squeezed through 8 layers of cheesecloth, which was previously washed with the buffer. The homogenate was centrifuged at 4000 g (max) for 5 min to obtain a pellet containing mostly whole chloroplasts, starch grains an

cellular debris. The supernatant was then centrifuged in a separate centrifuge tube at 10,000 g (max) for 20 min. The 10,000 g pellet contained mostly mitochondria. The remaining supernatant was designated the "supernatant". The 4000 g pellet was resuspended in 3.5 to 4.5 ml of 40 mM K phosphate (pH 7.6) and 2.5 mM DTT. The 10,000 g pellet was resuspended in 40 mM K phosphate (pH 7.6), 1.0 mM DTT and 0.01% (w/v) digitonin. Enzyme assays were as described above.

General Electrophoretic Procedures

Native gel electrophoresis. This procedure was performed according to Clark and Switzer (1977). The resolving gel was made by mixing 1 part stock A, 2 parts of stock C, 1 part water and 4 parts of 0.14% ammonium persulfate. Stock A was prepared by mixing 48 ml of 1 M HCl, 36.6 grams TRIZMA base (Sigma Chemical Co.), 0.23 ml TEMED (Sigma Chemical Co.) and water to a final volume of 100 ml. Stock C is 28 grams of acrylamide and 0.735 grams of N,N'-methylene-bis-acrylamide and water to 100 ml. Stock C was filtered through Whatman no. 1 filter paper and was stored in a dark bottle. Once poured, the resolving gel was overlayed with 2-butanol, until use. The stacking gel was prepared by mixing 1 part of stock B, 2 parts of stock D, 1 part of stock E and 4 parts stock F. Stock B is 9.35 ml of 1.0 M HCl, 1.2 grams of TRIZMA base, 0.1 ml TEMED and water to 20 ml. Stock D consisted of 2.0 grams acrylamide, 0.5 grams of N,N'-methylene-bis-acrylamide and water to 20 ml.

Stock E contained 1 mg riboflavin dissolved in 20 ml water. Stock F contained 40 grams of sucrose made to a final volume of 100 ml. The 10X buffer stock was made by mixing 6.0 grams TRIZMA, 28 grams glycine and water to 1 l. The final pH was approximately 8.3. An aliquot of protein was mixed with an equal volume of 0.002% bromophenol blue in 50% glycerol. The gel was subjected to electrophoresis at 200V. The gel was stained and fixed in methanol : acetic acid : water (5 : 1 : 5) and 0.25% Coomassie brilliant blue R 250 (Sigma Chemical Co.). The gel was destained in 7% acetic acid and 7% methanol.

Denaturing gel electrophoresis. This procedure was carried out according to Chua (1980). The resolving gel was prepared by mixing 7.5 ml of acrylamide stock, 6.0 ml of resolving gel buffer, 0.3 ml 10% SDS and 13.6 ml water. The mixture was degassed for 5 min under vacuum and then 14 µl of TEMED and 0.1 ml of 10% ammonium persulfate were added. The acrylamide stock was prepared by dissolving 7.5 grams acrylamide and 0.2 grams N,N'-methylene-bis-acrylamide in water to a total volume of 25 ml. The stock solution was filtered and stored in the dark at 4 ^{o}C. The stacking gel was prepared by mixing 2 ml of acrylamide stock, 2.5 ml of stacking gel buffer and 5.3 ml water. The stock solution was degassed and 5 µl of TEMED and 50 µl of 10% ammonium persulfate were added. The stacking gel buffer (5X) was made by mixing 6.55 grams TRIZMA base in 240 ml water and then

adjusting the pH to 6.1 with approximately 1 ml concentrated sulfuric acid. The upper reservoir buffer was prepared as a 20X stock solution. It was made by dissolving 99.2 grams of TRIZMA base with 28 grams boric acid dissolved in water to a volume 600 ml. The pH of the solution was adjusted to 8.64 with saturated boric acid at 25 °C. The final volume was 1 l. The lower reservoir buffer contained stacking gel buffer of single strength. A sample was prepared for electrophoresis by mixing with 5 volumes of cracking buffer. This buffer was 50 mM TRIS-HCl (pH 7.4); 1% 2-mercaptoethanol, 10% (v/v) glycerol, 1% SDS and 0.0005% (w/v) bromophenol blue. The sample was heated in boiling water for 5 min and applied. The gel was subjected to electrophoresis at 200V.

Immunological Techniques

Immunization procedure. Fd-dependent GOGAT was purified to greater than 95% purity from commercially obtained spinach, using the procedures described for **B. napus** fd-dependent GOGAT. Fd-dependent GOGAT was dialysed overnight against 50 mM K phosphate (pH 7.2) and 0.15 M NaCl. Approximately (100 ug) of protein was emulsified with an equal volume of Freund's complete adjuvant (Difco) and injected both subcutaneously and intramuscularly into male San Juan rabbits. Booster injections of 50-100 µg of protein emulsified in Freund's incomplete adjuvant were repeated at monthly intervals. Serum was tested for its ability to

inhibit fd-dependent GOGAT activity. Preimmune serum did not affect fd-dependent GOGAT activity.

Antibody preparation. Collected blood was kept at 20 $^{\circ}C$ for 30 min and was then stored at 4 $^{\circ}C$ overnight (Bigelis et al. 1981). Serum was collected by centrifuging the sample at 2,000 g for 10 min. The sera was then either stored frozen or protein precipitated by 55% $(NH_4)_2SO_4$ (2.91 grams 100 ml^{-1}). The pellet was washed with 1.75 M $(NH_4)_2SO_4$ until it was white. The pellet was then resuspended in 10 mM NaH_2PO_4 (pH 7.8), 0.1 mM EDTA and 1% glycerol, and dialysed overnight against the same buffer. The protein mix was centrifuged at 10,000 g for 10 min. The supernatant was then applied onto a DEAE-cellulose column (at 5 mls of DEAE-cellulose per ml of sera). The column was washed with 2 volumes of 20 mM K phosphate (pH 7.8). The immunoglobulin fraction does not absorb to the column. The fraction was collected and concentrated by $(NH_4)_2SO_4$ precipitation. Finally, the pellet was dissolved in 20 mM NaH_2PO_4 (pH 7.0) and 0.15 M NaCl and dialysed.

Immunoprecipitation procedure. Plant extracts were prepared by grinding 100 to 500 mg fw of leaves or roots in 250 to 1500 ul of I-grind buffer: 50 mM $NaHPO_4$ (pH 7.2), 0.2 M NaCl, 5 mM EDTA, 1 mM 2-mercaptoethanol, 0.02% NaN_3, 0.5% NP-40 (Sigma Chemical Co.) detergent and 20 mM KCl. BSA (0.5 mg ml^{-1}) was added after grinding. The extract was clarified

by centrifugation in an Eppendorf microcentrifuge for 10 min at 4 °C. The supernatant was incubated with 15 to 20 ul of partially purified antibody for 12 hr at 4 °C.

Staphylococcus aureus absorbent was prepared according to Kessler (1975). A 1/10 volume of absorbent was added to the antibody-plant extract and incubated for 2 hr on ice. The mixture was centrifuged for 15 s in an Eppendorf microcentrifuge and the pellet was washed with 50 mM Tris-Cl (pH 7.4), 0.15 M NaCl, 50 mM EDTA, 0.02% NaN_3 and 0.5% NP-40 (Bigelis et al. 1981). This procedure was repeated twice. The final pellet was resuspended in 1% SDS, 50 mM Tris-Cl (pH 8.0) and 1 mM EDTA. The sample was heated in boiling water for 2 min and centrifuged in an Eppendorf microcentrifuge for 5 s. The supernatant was applied to the gel.

The above procedure was used throughout the initial analysis of the **GLUS** mutants. However, a contaminating protein appeared to be nonspecifically appearing on the SDS-PAGE (see Results). Therefore, an alternative procedure was developed in consultation with Dr. Dan Geitz (Dept. of Genetics, Univ. of Alberta). Extracts were prepared as above, the antibody was added and incubation carried out for 3 to 4 hr at 4 °C. The absorbent was then incubated with the extract for 10 min at 4 °C. The extract was centrifuged for 15 s in the microcentrifuge. The pellet was washed once with 50 mM Tris-Cl (pH 7.8), 0.15 M NaCl, 1.2% Triton X-100, and 0.1 mM EDTA, once with 50 mM Tris-Cl (pH 7.8), 1.2 M KCl,

1.2% Triton X-100 and 0.1 mM EDTA and once with 10 mM Tris-Cl (pH 8.0). The final precipitate was extracted in DeNAT buffer.

CRM determination. The level of fd-dependent GOGAT CRM was measured by the ability of a mutant extract to protect the WT fd-dependent GOGAT activity from inhibition by the antibody. Plant extracts were prepared in I-grind buffer and aliquots of these extracts were incubated with varying concentrations of antibody for three hours. The extract was centrifuged and the amount of antibody in the supernatant was measured by its ability to inhibit WT fd-dependent GOGAT activity. By definition, the percentage of CRM concerns the amount of WT fd-dependent GOGAT activity remaining after the addition of antibody from a mutant extract incubation, over the initial activity of the WT strain x 100. **S. aureus** absorbent were then added to remove the antibody-enzyme complex. After centrifugation the supernatant was assayed for fd-dependent GOGAT activity.

Chloroplast isolation for CRM determination. Plants were grown in continuous light in a CO_2-enriched atmosphere (0.75 to 1.0% CO_2) and given 12-16 hours of darkness before the experiment. Protoplasts were used as a source for intact chloroplasts, as the yield is generaly higher than from direct grinds of leaf tissue (Somerville et al. 1981). Approximately 5 grams fw of leaves were harvested and the

leaves were sliced using an acetone-washed razor blade. Forceps were used to hold the material in a petri dish, which also contained the Proto-ISO-buffer: 0.5 M sorbitol, 10 mM MES, 1 mM $CaSO_4$, 1 mM KCl, 0.1% (w/v) BSA and 0.1% (w/v) PVP-40 (Sigma Chemical Co.), pH 5.5 at 22 °C. The leaves were sliced in widths of approximately 1.0 mm and leaves were washed once by vigorous stirring in the Proto-ISO-buffer. Throughout the entire procedure, a low light intensity of 25 uE m^{-2} s^{-1} was maintained on the leaves. The light was filtered through 10-cm of water. The slices were then infused (by applying a gentle vacuum to the tissue) with Proto-ENZ-buffer, which is the same as the Proto-ISO-buffer, except 0.9% (w/v) cellulase R-10 (Yakult Biochemicals), 0.5% (w/v) macerozyme (Yakult Biochemicals and 1.6% (w/v) cellulysin (Calbiochem, grade B) were added. Digestion of the cell walls was complete in 1.5 hr. After this time the slices were gently stirred for 5 min to release the protoplasts. This mixture was then passed through a 200 u nylon net. The protoplasts were chilled to 4 °C and pelleted by centrifugation for 4 min at 100 g and resuspended in cold 0.5 M sorbitol, 10 mM MES, 1 mM KCl and 0.1% (w/v) BSA, pH 6.2 at 4 °C. To this, Float-buffer was added in order to separate cellular debris from the protoplasts. This buffer contained: 0.45 M sorbitol, 60% Percoll (Pharmacia Fine Chemicals) (v/v), 10 mM MES (pH 6.2), 1mM KCl and 1 mM $CaCl_2$. The suspension was centrifuged at 100 g for 5 min. The banded protoplasts were collected

and diluted by 10 volumes of Proto-ISO-buffer and pelleted. The pelleted protoplasts were resuspended in Proto-Lysis-buffer: 0.3 M sorbitol, 20 mM Hepes (pH 7.8), 5 mM EGTA, 5 mM EDTA, 10 mM $NaHCO_3$, 0.1% (w/v) BSA and the suspension forced through a syringe that was blocked with a 15 u nylon net, (Telko, Inc.). Chloroplasts were collected by centrifugation for 40 s at 270 g and resuspended in Chloro-buffer: 0.3 M sorbitol, 20 mM Hepes (pH 7.8), 5 mM $MgCl_2$, 2.5 mM EDTA, 10 mM $NaHCO_3$ and 0.1% (w/v) BSA. The chloroplasts were stored on ice. This procedure is a modification of the one employed by Somerville at al. (1981).

Polyacrylamide gel electrophoresis of immunoprecipitates. Immunoprecipitates and total leaf polypeptides were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) which was performed as described by Chua (1980). Protein samples were denatured by incubating the sample in boiling water for 3 min, after the samples were mixed with 4-5 volumes of DeNAT buffer: 1% SDS, 10% glycerol, 50 mM Tris-Cl (pH7.5) and 1% DTT. The samples were centrifuged for 15 s in an Eppendorf microcentrifuge and the supernatants of the samples were layered on 7.5% gels. The gel was run at 35 ma for 8-10 hr. Once the bromophenol blue dye had migrated to near the bottom of the gel, the voltage was turned off and the gel was then stained. The staining solution was 0.25% Coomassie

blue R (Sigma Chemical Co.) in 50% methanol - 5% acetic acid. After 2-6 hr, the gel was placed in the destaining solution of 25% methanol - 10% acetic acid.

Electrophoretic separation of leaf polypeptides. A plant extract was mixed with an equal volume of ice-cold 10% trichloroacetic acid. The sample was incubated at 4 °C for 15 min and then centrifuged in an Eppendorf microcentrifuge for 2 min at 4 °C. The precipitate was washed three times with acetone to remove residual acid. The pellet was dissolved in DeNAT buffer and then heated in a boiling water bath for 3 min. Approximately 25 to 70 µg of protein was loaded per gel lane.

Enzymatic Analysis for RUBP

Extraction and determination of RuBP. RuBP was measured by following the incorporation of $^{14}CO_2$, using purified RuBISCO, into acid-stable product (Sicher et al. 1979). At appropriate time intervals, plants photosynthesizing in the IRGA were removed and killed by immersion in liquid N_2. Mature leaves (250 to 500 mg fw) were removed from immersed plants (3 to 5) and partially ground in a small mortar and pestle containing liquid N_2. The sample was then added to 2.0 to 3.5 ml of ice-cold 1.4 M $HClO_4$ in a Ten-Broeck homogeniser and ground thoroughly. The sample was removed to a microcentrifuge tube and centrifuged at 13,000 g for 5 min at 4 °C. The supernatant was decanted, the pellet extracted

with 350 to 500 µl of 0.4 M $HClO_4$ and centrifuged. The supernatants were pooled and MOPS was added to 35 mM, from a 4 M solution. The sample was neutralised to pH 7.0 to 7.5 with 4 M KOH. The $KClO_4$ was removed by centrifugation. Aliquots of the supernatant ranging from 0.15 to 2.0 ml were then added to RuBISCO-assay buffer, (as described above, except that 12.5 mM $NaH^{14}CO_3$ at 0.4 to 0.5 µCi / µmole was used) to give a final volume of 1.2 to 2.25 ml. The assay was initiated by adding purified *B. napus* RuBISCO (approximately 50 µg) and the reaction was terminated after 30 to 45 min with 0.4 ml of 4 M formic acid. The samples were dried and counted by liquid scintillation spectroscopy.

Purification of RuBISCO for determination of RuBP levels. The RuBISCO used to determine RuBP levels was isolated from *B. napus* ("Altex") leaves. Approximately 400 grams of leaves (midribs removed) were homogenised in 2 l of RuBISCO-grind-buffer: 50 mM Tris-Cl (pH 8.2 at 4 °C), 20 mM $MgCl_2$, 5 mM 2-mercaptoethanol. The homogenate was filtered through 6 layers of cheesecloth and centrifuged at 10,000 g for 30 min. The supernatant was then treated with solid $(NH_4)_2SO_4$ and a final 35-60% fraction was precipitated. The precipitate was collected by centrifugation at 10,000 g for 30 min, dissolved in 50 mM Tris-Cl (pH 7.8), 1.0 mM EDTA, 1 mM 2-mercaptoethanol and dialysed overnight against the same buffer. The protein mixture was applied to a column (5 x 75 cm) of DEAE-cellulose, which had been equilibrated with 10

volumes of 25 mM Tris-Cl (pH 7.8) and 0.1 mM EDTA. The column was washed with 1 to 3 volumes of equilibration buffer and the RuBISCO activity was then eluted using a gradient of 0.1-0.5 M NaCl in Tris-Cl (pH 7.8) and 0.1 mM EDTA. The peak fraction was precipitated with $(NH_4)_2SO_4$, dialysed against the DEAE-cellulose equilibration buffer and applied to a 2.5 x 50 cm Ultragel ACA 22 (Pharmacia Fine Chemicals) column. The column was equilibrated in 25 mM Tris-Cl (pH 7.8), 2 mM $MgCl_2$ and 0.1 mM Na_2·EDTA. A DEAE-sephadex column (2.5 x 30 cm) was then run, using a buffer identical to the DEAE-cellulose. Protein was eluted with a 1 l linear gradient of 0.0 to 0.5 M NaCl in 50 mM Tris-Cl (pH 7.8) and 0.1 mM EDTA. RuBISCO was precipitated with 60% $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 10,000 g for 45 min and stored in 55% $(NH_4)_2SO_4$. Before use the sample was dialysed overnight against three changes of 2 l of 50 mM Bicine (pH 8.3) and 0.2 mM Na_2·EDTA. This procedure is a modification of the one used by Paulson and Lane (1966).

Intact Plant Gas Exchange

Gas exchange. Gas exchange measurements were conducted using intact plants at a temperature of 20 °C. Individual plants were placed in moist vermiculite, in a chamber connected to an Infrared gas analyser (IRGA) (Analytical Development Co., type-225) in order to monitor the CO_2

concentration. The gas exchange curves to be presented latter are an average of individual plant CO_2 exchange rates. The analyser was calibrated every day with gases of known composition. Dry gas of the desired composition was humidified to 70% to avoid dessication of the plants. The circulation rate of the gas was 150 to 300 ml min^{-1}. The light source was a 500-W flood lamp. The light was filtered through a 10 x 10 cm infrared reflecting mirror (Orion Co.) and through 10 to 12 cm of flowing water before reaching the plant chamber. The irradiance at the plant surface was measured at 300 to 335 $\mu E\ m^{-2}\ s^{-1}$ (PAR), using a LI-1905 quantum sensor (Lambda Instruments). When photosynthetic CO_2 exchange was determined against light intensity, layers of cheesecloth were placed between the light source and the plant chamber. The flow rate in the chamber was electronically controlled by a mass flow controller (Tylar Corp.). The gas regime is given with each experiment. More details of the photosynthetic apparatus are given by Somerville and Ogren (1982b) and a detailed scheme of the IRGA system is given in McCourt (1983).

$^{14}CO_2$ Labeling of Leaf Metabolites

Photosynthetic $^{14}CO_2$ fixation. A closed gas circulation system was employed (Somerville and Ogren 1982b; McCourt 1983). Plants were removed from their pots and placed into a glass chamber, which fit over a rubber stopper. The rubber stopper had a small well, filled with moist vermiculite, to

contain the plant. The chamber was submerged 12 cm deep into a bucket of flowing water, connected to a water-bath circulator, which maintained the temperature at 20 ^{o}C. The plant chamber was connected by tygon tubing to a peristaltic pump and to a 500-ml erlenmeyer flask, which served as a reservoir of air during the $^{14}CO_2$ labeling period. In-line with the pump, was a flask containing 8% [illegible] acid. Initially, the system was open and con[illegible] a gas cylinder, containing a known level of CO_2 [illegible] (remaining N_2). The gas from t[illegible]linder was humidified to approximately 70% rela[illegible]dity before entering the plant chamber. The gas f[illegible]rate was approximately 400 to [illegible]0 $ml \cdot min^{-1}$. The system was allowed to equilibrate for 20 min in the dark before the 500 W flood lamp was turned on to initiate photosynthesis. The light [illegible] passed through an infrared reflecting mirror. The light intensity was approximately 375 to 400 $\mu E \cdot m^{-2} s^{-1}$ (PAR). The system remained open until a 2 to 3 min before introducing the $^{14}CO_2$. At this point, the system was closed, by disconnecting it from the humidifier and gas cylinder. Carrier free $NaH^{14}CO_3$ (5 to 20 μCi) was injected into the phosphoric acid and the peristaltic pump passed the liberated $^{14}CO_2$ throughout the system. The volume of the system was calibrated so that the CO_2 concentration declined by less than 10% during the course of the labeling period. After a predetermined period, the plants were killed by

immersion into liquid N_2. The gas regime is given with each experiment.

The Feeding of NH_4^+ to A. thaliania.

NH_4^+ feeding. Plants were removed from their pots and the roots rinsed with distilled water. Individual plants at a mature rosette stage were treated four at a time in the IRGA system. The tip of the main root was severed with a scalpel, in a buffer of 10 mM MES (pH 5.5) and 0.5 mM Na_2EDTA. NH_4Cl was added to 25 mM and the plants were incubated in a humid chamber for 30 to 90 min at 20 °C and low light (25 µE m^{-2} s^{-1} of PAR). This preincubation period was necessary in order to cull those plants visably stressed by the manipulation. While the uptake of NH_4Cl was not uniform, the relationship between photosynthetic inhibition and measured leaf NH_4^+ levels was found to be positively related (see Results).

U-^{14}C-Malate Feeding of Leaf Strips

Malate feeding. In the U-^{14}C-malate (Amersham) feeding experiment, 4-8 leaves (from 1 to 3 plants) were sliced thinly and incubated in the dark with a buffer of 20 mM MES (pH 5.7), 0.25 M sorbitol, 0.1 mM $CaSO_4$, 10 mM KCl, 1 mM $MgCl_2$ and 1-2.5 umoles of U-^{14}C-malate (0.5 µC_i/umole). The tissue was equilibrated with 21% O_2 and 340 ppm CO_2 (remaining N_2), by incubating the tissue in small serum capped erlenmeyer flasks. The flasks were occasionally

swirled to help circulate the gases within the flasks and after 30 min of incubation, the tissue was washed three times with the above buffer, minus the ^{14}C-malate. The flasks were then placed into an open gas exchange system and illuminated from below at 350 µE m^{-2} s^{-1} (PAR). The gas regime during the incubation in the light was 21% O_2 and 340 ppm CO_2 (remaining N_2). At the end of the incubation period, the leaf fragments were removed and frozen in liquid N_2.

Sample Extraction and Fractionation

Metabolite analysis. For the analysis of ^{14}C-labeled products, mature leaves were ground in a Ten-Broeck homogeniser containing chloroform: methanol : formic acid (12 : 5 : 1) (Bieleski and Turner 1966). The homogenate was centrifuged at 200 g for 5 min and the supernatant decanted. The residue was reground in 1.0 ml of chloroform : methanol: formic acid and centrifuged. The supernatants were combined and 0.5 to 0.75 ml chloroform and 0.8 to 1.2 ml of water was added. This mixture was vortexed and centrifuged to separate the phases. The original residue was reextracted with 2% methanol and 2% formic acid, centrifuged and the supernatant combined with the above aqueous phase. The aqueous phase-mixture was lyophilised. Approximately 100 µl of the organic phase was saved for chlorophyll determination, using the coefficients of MacKinney (1965). The remaining organic phase was dried at 65 °C. This insoluble fraction was

digested with a 700 ul solution of 8.6% (v/v) H_2O_2, 30% $HClO_4$ at 70 °C for 4 to 5 hr. The sample was then counted by liquid scintillation spectroscopy (Somerville and Ogren 1982b).

The aqueous extract was fractionated by ion-exchange chromatography, basically following the procedures outlined in Atkins and Canvin (1971) and Somerville and Ogren (1982b). The lyophilised extract was resuspended in 5.0 ml of water and duplicate 100 ul aliquots were taken for determining total radioactivity. The samples were kept at 4 °C, until just before chromatography. Dowex-50 (Sigma Chemical Co.) (H^+) columns were used to remove the amino acids (basic fraction) from the extract. The resin was prepared according to Clark and Switzer (1977). Following removal of the fines, the resin was heated in 2M HCl with constant stirring. When the temperature reached 100 °C, the resin was allowed to settle and the yellow supernatant was poured off. This procedure was repeated using distilled water until the supernatant was clear. Subsequently, water was passed through the resin until the wash water pH was neutral after which the resin was poured into a pasteur pipette, plugged at the bottom with glass wool. The final column size was approximately 0.5 x 4.0 cm. The lyophilised extract, prepared as above, was added to the column and washed with 6 ml of water. The amino acids were then were eluted with 10 ml of 2 M NH_4^+OH. In order to test for recovery of amino acids (known to be important in

photorespiratory metabolism) approximately 200,000 dpm of each amino acid was added to a non-radioactive plant extract while still in the Ten-Broeck homogeniser. The amount of ^{14}C recovered after chromatography (as described below) was compared to the initial amount added. Recovery of glycine, serine and glutamate was in the 87.5-98% range. Glutamine recovery was about 70-80%.

In order to separate the organic acids from the phosphorylated compounds, and from sucrose, the remaining extract was passed over a Dowex-1 (formate) column. The Dowex-1 resin was prepared as above, except that after the HCl heating and decanting, the resin was heated in 2 M formic acid to 70 ^{o}C. The resin was washed until neutral in pH. The organic acids (mainly malate, succinate, glycolate and glyoxylate) were selectively eluted from the Dowex-1 column with 10 ml of 1.5 M formic acid (acid-1 fraction), sugar monophosphates (SMP; mainly PGA, R5P, fructose 6-phosphate, 6-phosphogluconate and glucose 6-phosphate) were eluted with 10 ml of 8 M formic acid (acid-2 fraction), followed by the bisphosphates (mainly RuBP, PEP and FBP) elution in 8 ml of 4 M HCl (acid-3 fraction). All three fractions were lyophilised and subjected to chromatography as described below. The recovery of ^{14}C-malate was tested by adding a known amount of ^{14}C-malate to a non-radioactive plant extract homogenisation. After chromatography of the acid-1 fraction (as described below) and the determination

of radioactivity, the recovery for malate was 85 ± 5% (average of 3 determinations ± S.E.). In a similar experiment, the recovery of ^{14}C-2-oxoglutarate, was 76 ± 13% (average of 3 determinations ± S.E.). The values presented were not corrected for yield.

The individual components of the fractions were separated by thin layer chromatography using MN 300 (Analtech) or descending paper chromatography (Whatman #3). The general procedures follow those outlined in Benson et al. (1950) and in Clark and Switzer (1977). The solvent for separating the amino acids was either n-butanol : acetone : diethylamine : water (10 : 10 : 2 : 8) or [illegible]l : water : pyridine (5 : 2 : 1) (Cossins and Sinha 1966). The solvent for separating the organic acids was either anhydrous diethylether : formic acid : water (7 : 2 : 1) or chloroform : methanol : formic acid (80 : 1 : 1) (Smith 1958) or n-butanol : propionic acid : water (372 : 180 : 245) (Benson et al. 1950). Phosphates were separated by 2-dimensional TLC chromatography using methanol : NH_4OH : water (7 : 1 : 2) in the first direction and formic acid : methanol : water (8 : 5 : 0.5) in the second direction (Davidson and Drew 1966) or the n-butanol-propionic acid-water was used in the first direction and and water-saturated phenol in the second (Benson et al. 1950). If only PGA was to be determined, descending chromatography with n-butanol-propionic acid-water was used (Benson et al. 1950). All chromatographs were dried, sprayed with autoradiograph enhance (NEN Enhancer)

and autoradiographed at -70°C. Radioactive areas were visualised on X-ray film (Kodak X-Omat AR). The radioactive areas were removed and directly placed in the liquid scintillation cocktail, and counted. Standards were individually chromatographed by one-dimensional chromatography and then metabolites developed by for 2-dimensional chromatography, were identified by comparison to a separate chromatograph. The phosphates were identified using a molybdate procedure (Davidson and Drew 1966). Organic acids were identified using a bromophenol blue reagent (Somerville and Ogren 1982b). The amino acids were identified with a ninhydrin reagent (Somerville and Ogren 1982b).

Ammonia determination. NH_4^+ was assayed immediately and directly from plant extracts using an NH_4Cl calibration curve (McCullough 1967). Approximately 75 to 150 mg fw of tissue was homogenised in 2.0 to 3.0 ml of chloroform : methanol : water (12 : 5 : 3). The sample was centrifuged at 200 g for 5 min and the supernatant decanted. The sediment was reground in 500 ul of methanol : chloroform : water and centrifuged. The supernatants were pooled and 1.5 ml chloroform and 1.0 ml water added, the sample vortexed and the phases separated by centrifugation at 200 g for 5 min. An aliquot (50 to 750 µl) from the aqueous phase was mixed with 5 ml of reagent A (5 gram phenol plus 25 mg of Na-nitroprusside dissolved in 500 ml water). Then 2.5 ml of

reagent B (1.25 grams NaOH combined with 2.1 ml Na-hypochlorite and diluted with water to a final volume of 250 ml) was mixed in. Color development took about 30 min at 20 °C. The absorbance was read at 625 nm.

Amino acid analysis. Amino acid analysis was performed on extracts of approximately 200 mg fw of leaves obtained from 3 to 4 plants killed in liquid N_2 and homogenised in methanol : chloroform : water (12 : 5 : 3) (Bieleski and Turner, 1[illegible]6). Leaves were homogenised in 2 ml of the methanol : chloroform : water mixture and centrifuged at 1000 g for 5 min. The supernantant was decanted and saved, and the sediment was re-extracted with 2 ml of 80% ethanol, centrifuged at 1000 g for 5 min, and the supernatant decanted. To the first supernatant, 0.5 ml of chloroform and 0.8 ml of water were added and the sample was mixed vigorously. The sample was centrifuged in a clinical centrifuge at 1000 g for 5 min. Approximately 1.5 ml of the aqueous phase was removed and saved. To the remaining portion of the sample, 1.0 ml of water was added, mixed vigorously and centrifuged. Approximatley 1.5 ml was removed and added to the previous aqueous phase. The aqueous phase was combined with 0.5 ml chloroform and 0.[illegible]5 ml of water, and centrifuged. At this point, an aliquot (100 to 350 µl) of the organic phase was removed, brought to a final volume of 10 ml with methanol, incubated in the dark at 0 °C for 30 to 60 min, and chlorophyll content was measured as a

function of the absorbance at 660 and 665 nm (Mackinney 1941). The remaining aqueous phase was combined with the 80% ethanol supernatant and dried under N_2 gas. The sample was resuspended in 500 µl of water and the amino acids were isolated by Dowex-50 (H^+) chromatography. The eluted amino acids were frozen and then lyophilized. At this point the samples were given to Mr. M. Natriss (Dept. of Biochemistry, Univ. of Alberta) who determined amino acid levels using a Beckman 119CL amino acid analyser. The amounts of glutamine and asparagine were estimated from the increase in glutamate and aspartate, respectively, after hydrolysis of the sample in 6 N HCl for 12 hr at 110°C.

Measurements for the efficiency of recovery were done with glutamate, glutamine and glycine. Duplicate amino acid samples were prepared. One of the duplicates was treated as above and the final concentration determined by amino acid analysis. The second duplicate was added directly to a leaf sample in methanol : chloroform : water before grinding. The leaf sample was from the WT strain kept in the dark. The percent recovery was determined as the difference between the amount of the amino acid found in the control leaves and the amount of that amino acid in leaves plus the added amino acid, over the added amino acid added alone to the methanol : chloroform : water mixture. In 2 experiments, the average percent recovery for glutamine was 82 ± 4, for glutamate 87 ± 7, and for glycine 93 ± 3.

Determination of starch. The following method is according to a protocol originating from G. Peiser (formally Univ. of Illinois; pers. com.) which is similar to published information by Stitt et al. (1978). Approximately 500 mg of leaves were ground in 7.5 ml of methanol : chloroform : water (12 : 5 : 3) and centrifuged (30,000 g for 20 min). Subsequently, the sample was extracted with methanol : chloroform : water and centrifuged. The insoluble residue was dried overnight at 37°C (to remove chloroform). The resulting pellet was shaken in 0.1 ml 50% ethanol, diluted with 2.0 ml of water and autoclaved for 120 min at 121°C. After cooling, 2.0 ml of 0.2 M sodium acetate (pH 4.8), 2.0 ml of 0.5% (w/v) amyloglucoside (Daizyme; Miles Laboratories) and 0.1 ml of 0.01% (w/v) alpha-amylase (Sigma Chemical Co.) were added and the sample incubated for 12 to 16 hr at 37 °C. The amyloglucosidase was initially prepared in water, centrifuged (25,000 g for 40 min) and the supernatant precipitated with 55% acetone at −15 °C. Aliquots (10 to 300 ul) from this starch digest were diluted with water to a volumne of 1.0 ml. 1.0 ml of a mixture (25 parts copper reagent A and 1 part copper reagent B) was added to each sample and the sample boiled for 30 min. 250 ml of reagent A contains: 6.25 grams of anhydrous $NaCO_3$, 6.25 grams K:Na-tartrate, 5.0 grams $NaHCO_3$ and 50 grams anhydrous Na_2SO_4. 100 ml of reagent B contains: 15 grams $CuSO_4$ $5H_2O$ and 2 drops H_2SO_4. After the samples were cooled

in water, 1.0 ml arsenomolybdate reagent (Sigma) was added to the sample. After mixing the absorbance at 500 nm was measured. Glucose standards of 10, 20, 40, 60 and 75 µg were used.

Measurement of Photosynthesis in Intact Chloroplasts and in Thylakoids

Electron transport rate measurements. Four hundred mg of leaves were homogenised for four 5 s bursts in a blender at low speed. The blender contained 15 ml of 67 mM KH_2PO_4/NA_2HPO_4 (pH 7.6), 0.4 M sorbitol, 50 mM NaCl and 0.2% (w/vol) BSA. The homogenate was filtered through a 25 µ nylon net and centrifuged at 1,000 g for 5 min. The thylakoids were isolated by rupturing the chloroplasts in 1.0 ml of homogenisation buffer, minus sorbitol, but with 5 mM $MgCl_2$. The suspension was centrifuged at 2,800 g for 5 min and the thylakoid pellet was resuspended in 250 µl of the above phosphate buffer and NaCl (modified from McCourt 1983). Thylakoids were kept on ice and under dim light until use. Photosystem II rates for electron transport from water to ferricyanide were determined by measuring O_2 evolution. The water to ferricyanide ($K_3Fe[CN]_6$) measurements were made at 20-22°C using an O_2 electrode (Hansatech Ltd.) similar to Delieu and Walker (1976). Illumination was provided by a projector lamp fitted with a Corning 2-62 red glass filter. Illumination was measured at the cuvette surface to be 800 µE m^{-2} s^{-1} (PAR). After 30 s 20 µl of 0.5 M NH_4Cl was added

to the 1.0 ml reaction mix of: 50 mM HEPES (pH 7.6), 1 mM $MgCl_2$, 1 mM Na_2EDTA, 1.5 mM $K_3Fe(CN)_6$ and approximately 18.5-25 µg chlorophyll (Powles and Critchley 1980). For whole chain photosynthetic electron transport (H_2O to PS1) an assay system similar to the ferricyanide one was used, with the exception that 4.0 mM NaN_3, 0.1 mM methyl viologen and 10 mM $MgCl_2$ were used and O_2 consumption was measured (Powles and Critchley 1980).

Intact chloroplast photosynthesis. In order to obtain a high percentage of intact chloroplasts, chloroplasts were isolated using protoplasts (see Immunological Techniques). The chloroplast pellet was resuspended in 0.33 M sorbitol, 20 mM Hepes (pH 7.6), 5 mM $MgCl_2$, 25 mM EDTA, 10 mM $NaHCO_3$ and 0.1% BSA (fatty-acid free, Sigma Chemical Co.) and stored on ice (Somerville et al. 1981). A typical assay medium was 0.33 M sorbitol, 1.0 mM EDTA, 0.1% BSA, 0.1 mM KH_2PO_4 and 200 units ml^{-1} catalase (Somerville et al. 1981). The optimum KH_2PO_4 concentration for maximum rates was individually determined each time, for each strain. Chloroplast intactness was determined by ferricyanide-dependent O_2 evolution before and after shock (Lilley et al. 1975).

Other Methods

Starch gel electrophoresis. This procedure was performed under the guidance of Dr. Z. F. Florence (Dept. of

Forrest Genetics, Univ. of Alberta). Plants were raised in a high CO_2 growth chamber. The procedure is according to Yeh and O'Malley (1979).

Chlorophyll determination. Unless specifically noted for a specific technique, chlorophyll was determined at 645, 652 and 663 nm (Bruinsma 1963). Aliquots of 10 to 250 µl were removed from leaf homogenates and were diluted to 4.0 ml with water and acetone to make a final concentration of 80% acetone. The sample was left in the dark at 4 °C for 30 to 60 min before measuring the absorbancy. The chlorophyll content (µg / ml) was calculated by the following two formula:

$$chl_{a+b} = 20.2\ (ABS_{645}) + 8.0\ (ABS_{663})$$
$$chl_{a+b} = 27.2\ (ABS_{652})$$

The determination of protein concentration. Protein was estimated according to Lowry et al. (1951) with lysozyme as a standard in the GOGAT isolation experiments. Fresh alkaline copper reagent was made by mixing 1 ml of 1% $CuSO_4$ $5H_2O$ with 1 ml of 2% sodium tartrate and 98 ml of 2% Na_2CO_3 (in 0.1 N NaOH). Exactly 6 ml of the copper reagent was added to a protein solution of volume 1.2 ml. This was mixed immediately and after 10 min 0.3 ml Folin-Ciocalteau reagent was added and vortexed. After 30 min, the absorbance was determined at 500 nm against a water control. For the determination of standard protein concentrations: 10, 25,

50, 75, 100 and 200 ug protein were employed.

Dye-binding assay for protein. For the determination of protein during the immunological studies, the Coomassie G-250 (Sigma Chemical Co.) dye binding assay was used (Spector 1978). For the preparation of the reagent, 100 mg of Coomassie were dissolved in 50 ml of 95% ethanol. Then 100 ml of 85% phosphoric acid was added. This was diluted with water to 1 l and filtered through Whatman 3 mm filter paper. For most leaf tissue grinds, 25 to 100 ul of supernatant was added to 0.4 ml buffer and subsequently either 10 or 50 or 75 µl of this was added to 140 or 110 or 95 µl of water in an acid cleaned glass tube. 1.0 ml of Coomassie reagent was added, mixed by vortexing and allowed to calibrate for 3 min. The absorbance was read at 595 nm. A BSA stock (0.25 mg / ml) was diluted into the same grind buffer as the leaf material was homogenised in, and a standard curve of 10 to 25 ug BSA was developed.

Protein determination with deoxycholate. It has been that the standard Lowry method for determining protein is not reliable in the presence of many commonly used chemicals (Spector 1978). In the opinion of the present author there is a tendency for the Coomassie-dye to precipitate and yield varied protein measurements with crude extracts. Therefore, the procedure of Bensadoan and Weinstein (1976) was eventually used, yielding reliable results (data not included). This assay was used for the experiments

concerning the determination of RUBP, RuBISCO activity and experiments concerning revertants.

A 10-200 ul aliquot from a leaf homogenate was diluted with water to 1.0 ml and sodium deoxycholate was added to 200 µg/ml. The mix was inverted several times and incubated at 20°C for 15 min. To the approximate 1.1 ml mix, 330 µl of 24% trichloroacetic acid was added and the mix was inverted several times. The mixture was centrifuged for 10 min in a Eppendorf microcentrifuge. The supernatant was removed with a pipette tip. A 50 : 1 reagent of 2% Na_2CO_3 (w/vol) and 0.5% $CuSO_4 \cdot 5H_2O$ (w/vol), plus 1.0% sodium citrate was prepared and 1.0 ml of the reagent was added to the TCA pellet. 100 µl of freshly prepared and diluted (water) 1 : 2 Folin-Ciocalteu reagent was added. After mixing the color reaction was allowed to develop for 45 min in the dark at 20°C. The absorbance was read at 660 nm. A BSA solution was used as a standard.

Liquid scintillation techniques. The scintillation cocktail used consisted of 500 ml of toluene, 250 ml of Triton X-100 and 3 grams of 2,5-diphenyloxazole (Anderson and McClure 1973). A Beckmann LS counter was used.

Results

Isolation of GOGAT Proteins from B. napus

Table 1 lists the GOGAT activities found in **B. napus** leaf and root issue. This species was used in order that plenty of tissue would be available for the isolation of the respective enzymes. Using four different reductants, four enzyme activities were found. In crude extracts of 10-day old etiolated leaves, fd-dependent GOGAT activity was measured at 0.21 µmoles glutamate mg protein^{-1} hr^{-1}. However, upon illumination, fd-dependent GOGAT activity increased within 1 hour to a value of 1.3 and to 7.6 after 72 hours of illumination (Table 1). There was a slight decrease in NADH-dependent GOGAT activity after illumination of the etiolated leaf. It appears therefore that in the leaf light affects the expression of these two enzymes differently. As mentioned in the Introduction, NADPH-dependent GOGAT has been reported in both tobacco and soybean cell cultures (Chia and Shargool 1979; Suzuki et al. 1984). NADPH-dependent activity was detected in DEAE-cellulose extracts in **B. napus** (not shown). However, this activity was not recovered following ACA-34 column chromatography (not shown). One possible explanation for the latter observation, is that the NADPH-dependent activity may not be a unique protein, but may simply reflect the presence of a second protein utilising the NADPH to form NADH in

crude leaf extracts. Therefore, in crude extracts the NADPH-dependent activity may actually represent NADH-dependent GOGAT activity. For the leaf tissue, fd- and NADH-dependent GOGAT activities were localised to the pla[illegible]tion (Table 2).

Data for a typical purification of the root and leaf NADH-dependent GOGAT's are shown in Table 3. Yields amounted to approximately 20% in each case. Throughout the purification procedures, the two enzymes had comparable elution profiles (not shown). During purification the presence of glycerol and DTT were found to keep the loss of NADH-dependent GOGAT activity to a minimum (not shown). The purity of the resulting proteins were assayed by native PAGE. The NADH-dependent GOGAT proteins upon electrophoresis moved to identical positions in the gel and yielded a single band (Plate 1).

The purified NADH-dependent GOGAT from both root and leaf tissue yielded a final specific activity in excess of 20. Both enzymes were specific for NADH, and demonstrated no activity with NADPH, methyl viologen or fd (not shown). In order to examine as to whether the purified enzymes utilise alternative substrates, or if other substrates, affect the GOGAT activities, assays were performed after incubating the enzymes with the appropriate metabolite. No activity was detected when using $(NH_4)_2SO_4$, glutamate, asparagine or oxaloacetate as substrates (not shown). Malate, PEP,

Table 1

Glutamate synthase activities from the root and leaf tissue of *B. napus*. The experiment was done twice and the values were averaged (± S.E.).

Tissue	reductant	GOGAT Activity: 10 day etiolated	GOGAT Activity: 3 days illumination after 10 days dark growth
		(μmoles mg protein^{-1} hr^{-1})	
Leaf			
	fd	0.21±0.06	7.60±0.80
	methyl viologen	0.10±0.02	3.45±0.30
	NADPH	0.24±0.04	0.30±0.10
	NADH	0.60±0.05	0.52±0.20
Root			
	fd	0.46±0.10	0.45±0.20
	methyl viologen	0.21±0.03	0.12±0.08
	NADPH	0.38±0.20	0.49±0.40
	NADH	0.89±0.90	1.20±0.90

Table 2

Distribution of GOGAT activities within the leaf of B. napus. The experiment was replicated two times and the values were averaged. The values represent the percent of total activity. The recovery of the initial activity, is the sum of the activity determined in the three fractions, over the activity in the initial crude extract (before centrifugation).

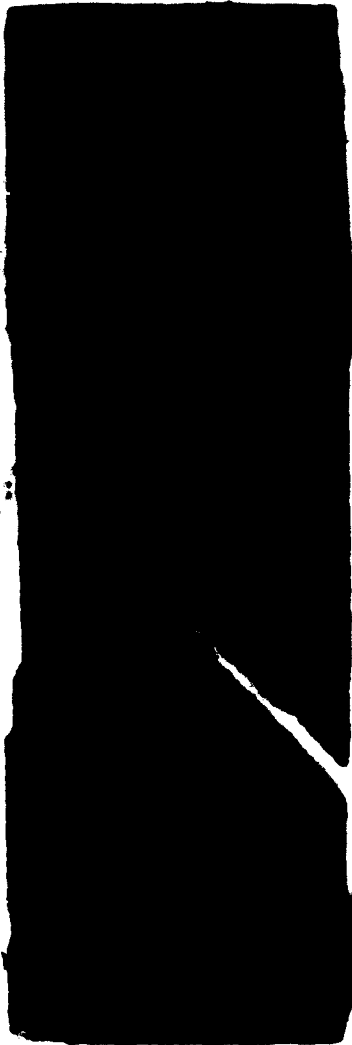
Fraction	Recovered Activity					
	fd-GOGAT	NADH-GOGAT	RuBISCO	Chl	Catalase	Cytochrome C oxidase
4000 g pellet	93.6	90.0	91.4	98.7	5.0	3.2
10000 g pellet	0.0	1.5	0.6	0.8	24.4	93.2
supernatant	6.2	8.0	8.4	0.3	68.1	2.8
recovery of initial activity (%)	99.0	93.0	90.0	112.0	106.0	82.5

Table 3

Purification of NADH-GOGAT from root and leaf tissue of *B. napus*.

Source	Protein (mg)	Activity (units)	Specific activity (U/mg)	Yield (%)
Leaf				
supernatant	6850	143.9	0.021	100
$(NH_4)_2SO_4$	5152	128.8	0.025	89
DEAE	256	102.1	0.400	71
ACA-34	1.7	34.4	20.100	23
Root				
supernatant	2910.0	174.6	0.06	100
$(NH_4)_2SO_4$	1118.0	156.6	0.14	89
DEAE	63.3	100.0	1.60	57
ACA-34	2.0	42.6	21.20	24

Plate 1 Nondenaturing PAGE of the NADH-dependent GOGAT's purified as described in the text. The protein in lane 1 is from leaves and in lane 2 from root tissue. Approximately 10 ug of protein was added in each lane. The gel was run from top to bottom.


- NADH GOGAT
A B

alanine, phosphogluconate, aspartate or 3-phosphoglycerate were tested for their effect on enzyme activity. At a concentration of 5 mM, they did not affect NADH-dependent GOGAT activity (not shown). The plot for the estimated MW of the root NADH-dependent GOGAT is given in Figure 2. The estimated MW was 225,000. The optimum pH was 7.4 to 7.6 (Table 4), which is similar to the NADH-dependent GOGAT isolated from pea (Matoh et al. 1980), but lower than the pH optimum of 8.5 reported for the enzyme from lupin nodules (Boland and Benny 1977). The Km's for NADH were difficult to determine due to the low activity at very low NADH concentrations. The results suggest however that the Km's were similar (Fig. 3) and comparable to the Km of 4 uM as determined for the pea enzyme.

NADH-dependent GOGAT is a multisubstrate enzyme, and Lineweaver-Burke double reciprocal plots were used at varying substrate levels. A primary plot for root NADH-dependent GOGAT at varying glutamine and fixed 2-oxoglutarate is shown (Fig. 4). The plot shows the converging slopes characteristic of a sequential enzymatic mechanism (Piszkiewicz 1977). Subsequently. the intercepts were plotted against the inverse of the particular concentration (Fig. 4.) The kinetic data for both enzymes are summarized in Table 4 and the respective plots in Figures 5, 6 and 7. Boland (1979) has shown that NADH binds first to the enzyme (possibly inducing a conformational change) followed with a random-order binding of glutamine

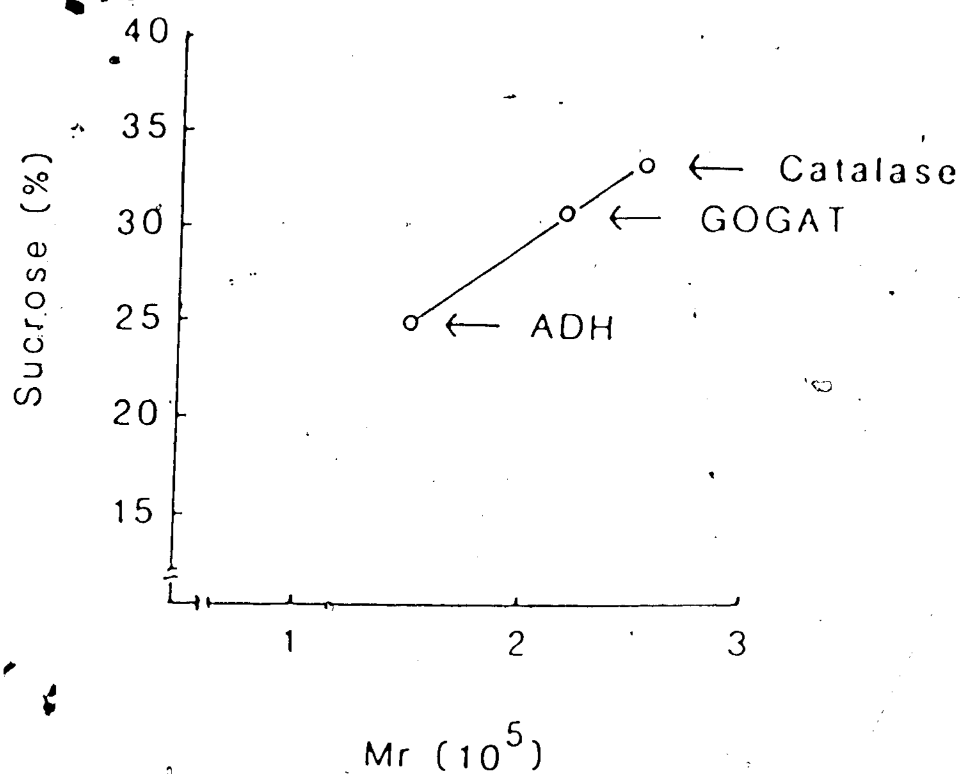


Figure 2 Determination of native MW for root NADH GOGAT by centrifugation on sucrose gradients. The position of the protein is plotted against MW. The MW for catalase is 250,000 and for ADH is 140,000.

Table 4

Properties of NADH-GOGAT from root and leaf tissue of **B. napus**.

Source	Apparent MW	K_m (um)			pH optimum
		NADH	2-OG	GLN	
Root	220,000-235,000	4.5	80	590	7.4-7.6
Leaf	225.000-240,000	4.0	80	625	7.4-7.6

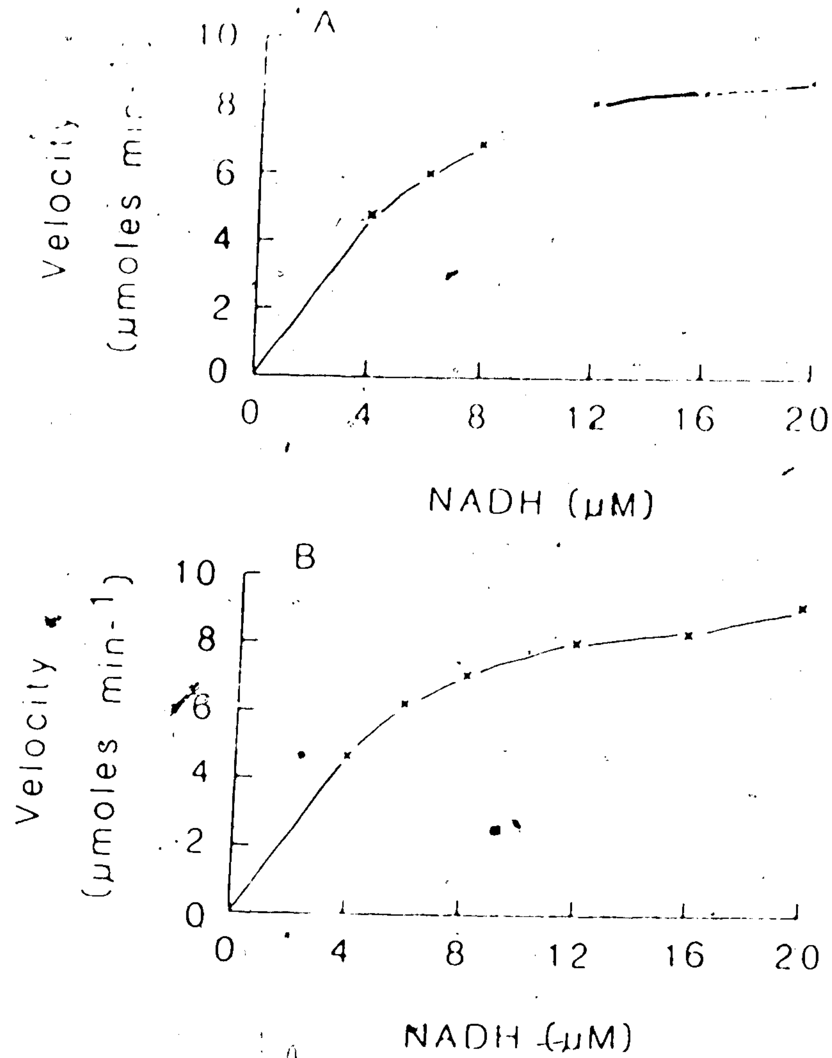


Figure 3 Plots of NADH-dependent GOGAT activity versus NADH concentration from leaf (A) and root (B) tissues.

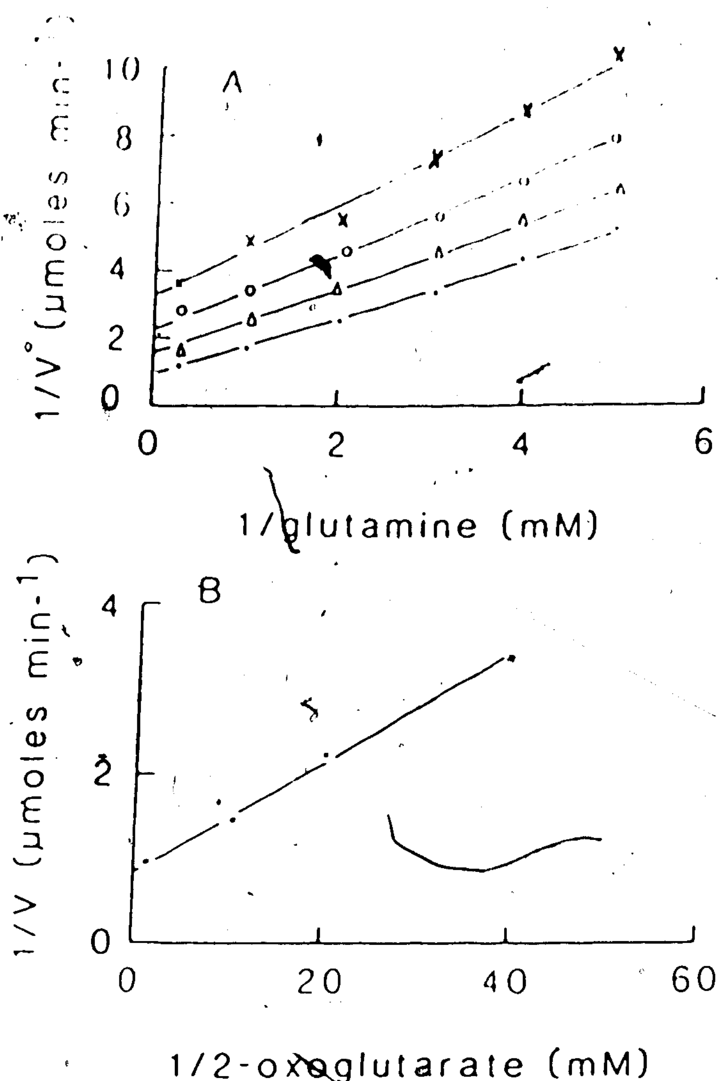


Figure 4 A. Plots of 1/v versus 1/[glutamine] at constant concentration of 2-oxoglutarate for root NADH-dependent GOGAT. The 2-oxoglutarate concentrations were: x =500 uM; o =100 uM; Δ =50uM; • =25 uM. B. Plot of intercepts of plots of 1/v versus 1/[glutamine] at fixed concentrations of 2-oxoglutarate versus 1/[2-oxoglutarate].

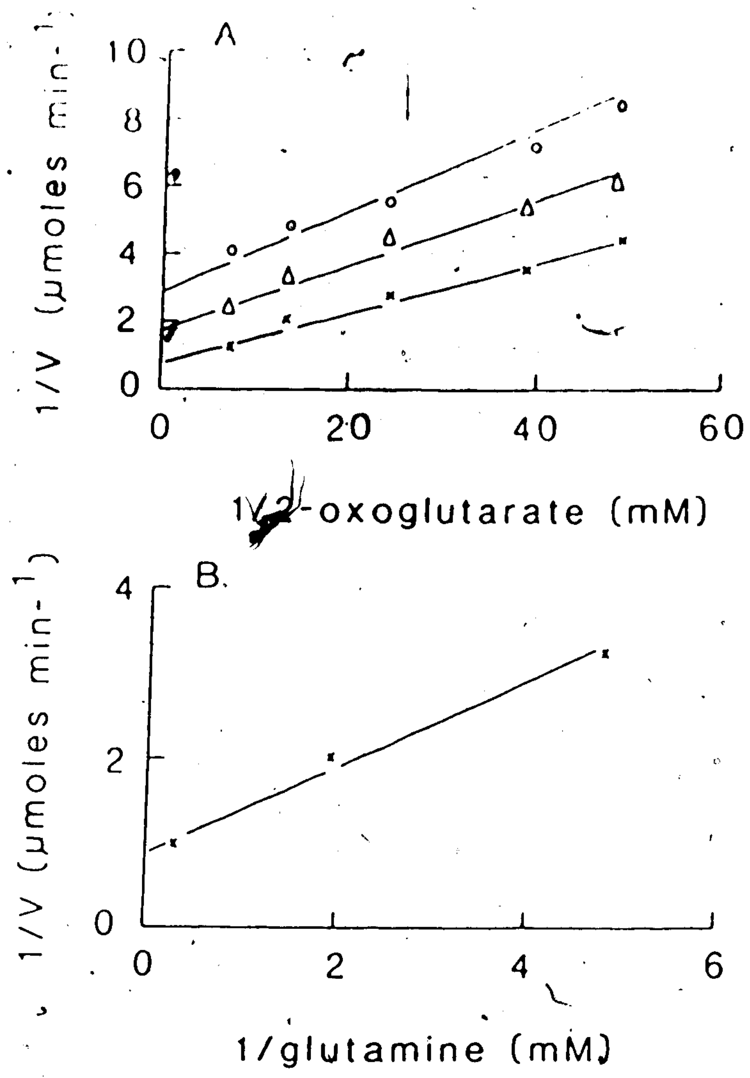


Figure 5 A. Plot of 1/v versus 1/[2-oxoglutarate] at constant concentrations of glutamine for root NADH-dependent GOGAT. The glutamine concentrations were: o =0.2 mM, Δ =0.5mM; x =2.5mM. B. Plot of intercepts of plots of 1/v versus 1/[2-oxoglutarate] at fixed concentration of glutamine versus 1/[glutamine].

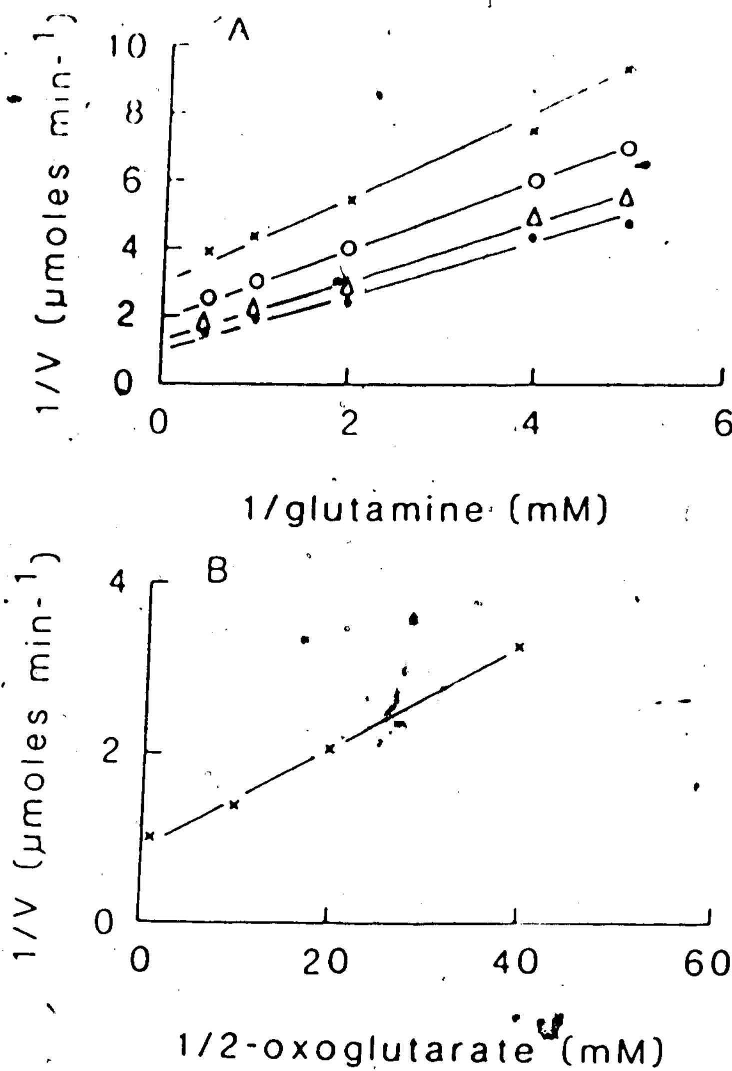


Figure 6 A. Plot of 1/v versus 1/[glutamine] at constant concentration of 2-oxoglutarate for leaf NADH-dependent GOGAT. The 2-oxoglutarate concentrations were: ● =1000uM; Δ =100 uM; o =50 uM; x =25 uM. B. Plot of intercepts of plots of 1/v versus 1/[glutamine] at fixed concentration of 2-oxoglutarate versus 1/[2-oxoglutarate].

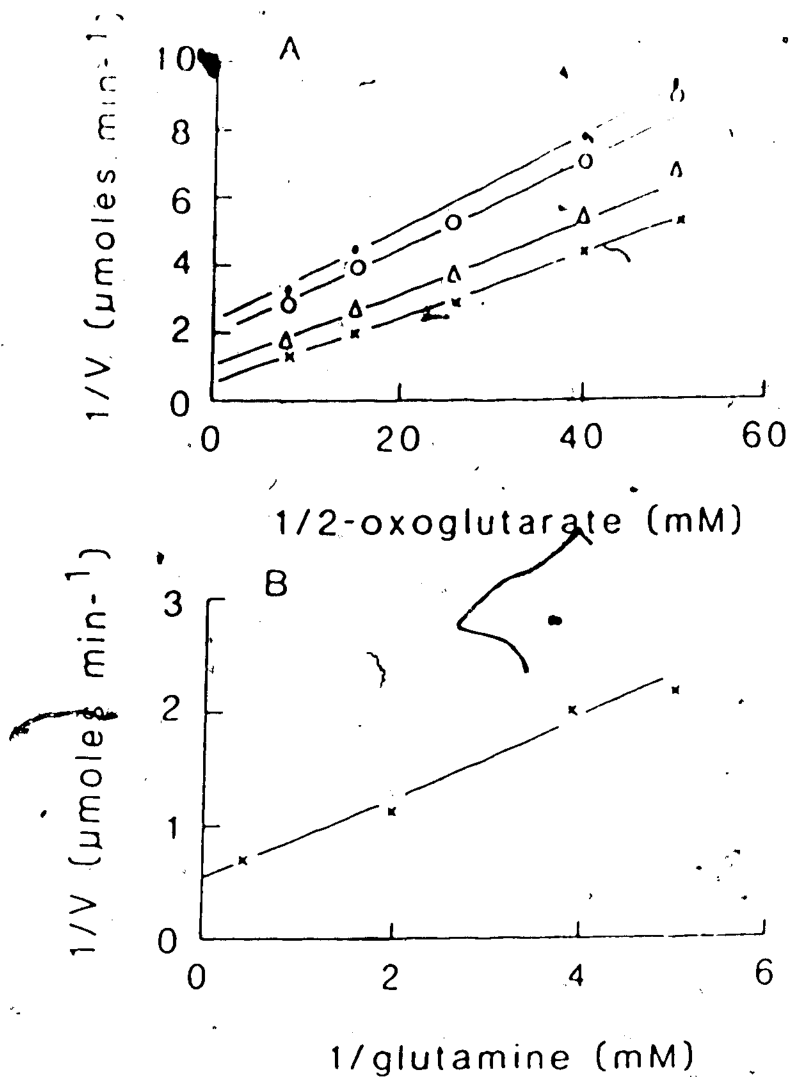


Figure 7 A. Plot of 1/v versus 1/[2-oxoglutarate] at fixed concentration of glutamine for leaf NADH-dependent GOGAT. The glutamine concentrations were: ● =0.2 mM; o =0.25 mM; Δ =0.5 mM; x =2.5 mM. B. Plot of intercepts of plots of 1/v versus 1/[2-oxoglutarate] at fixed concentrations of glutamine versus 1/[glutamine].

and 2-oxoglutarate. Since the double-reciprocal plots showed a convergence below the horizontal axis (in support of Boland's (1979) work) it is apparent that the concentration of one substrate, can influence the Km for the second substrate in the GOGAT catalysis reaction (Piszkiewicz 1977).

Leaf fd-dependent GOGAT was purified to homogeneity with a final specific activity of 42 U mg protein^{-1} (Table 5). The enzyme had no activity with aspartate, oxaloacetate or NH_4^+ as substrates (not shown). Potassium, sodium and NH_4 ions were not found to be stimulatory, and 10 mM $MgCl_2$ yielded a 15% inhibitory effect (not shown). At 5 mM PEP, malate, phosphogluconate, fructose 6-phosphate, alanine and aspartate did not inhibit the activity (not shown). The pH optimum was between 7.5 and 8.0, which is considerably higher than the pH optimum of 7.3 reported for the fd-dependent GOGAT from V. **faba** (Wallsgrove et al. 1977) and for the one from rice (Suzuki and Gadal 1982). The apparent MW was found to be 145,000 in denaturing gels (Plate 2). The native MW was determined with sucrose gradients. In sucrose gradients, fd-dependent GOGAT sedimented almost identically with yeast ADH (Fig. 8). Yeast ADH is reported to have a MW of 150,000 and an S value of 6.7. Consequently the MW of native fd-dependent GOGAT amounts to approximately 150,000. Therefore, fd-dependent GOGAT of B. **napus** was considered to be active as a single subunit. In rice, fd-dependent GOGAT was reported to be active as a dimer (Suzuki and Gadal

Table 5

Summary of leaf fd-GOGAT purification.

Fraction	Protein (mg)	Activity (units)	Specific Activity (U/mg)	Yield (%)
supernatant	7330	733	0.10	100
$(NH_4)_2SO_4$	5200	676	0.13	92
DEAE	620	502	0.81	68
ACA-34	22.3	325	14.60	44
hydroxylapatite	5.1	214	42.10	29

Plate 2 Results of SDS-PAGE performed on **B. napus** leaf fd-dependent GOGAT and purified as described in the text. The gel photographed contains approximately 8 ug protein in lane 2, stained with Coomassie brilliant blue R. The MW standards are in lane 1 and were added at 15-20 ug per protein. The direction of migration was from top to bottom.

200,000-
116,000-
94,000-
68,000-
43,000-
- fd-GOGAT
A
B

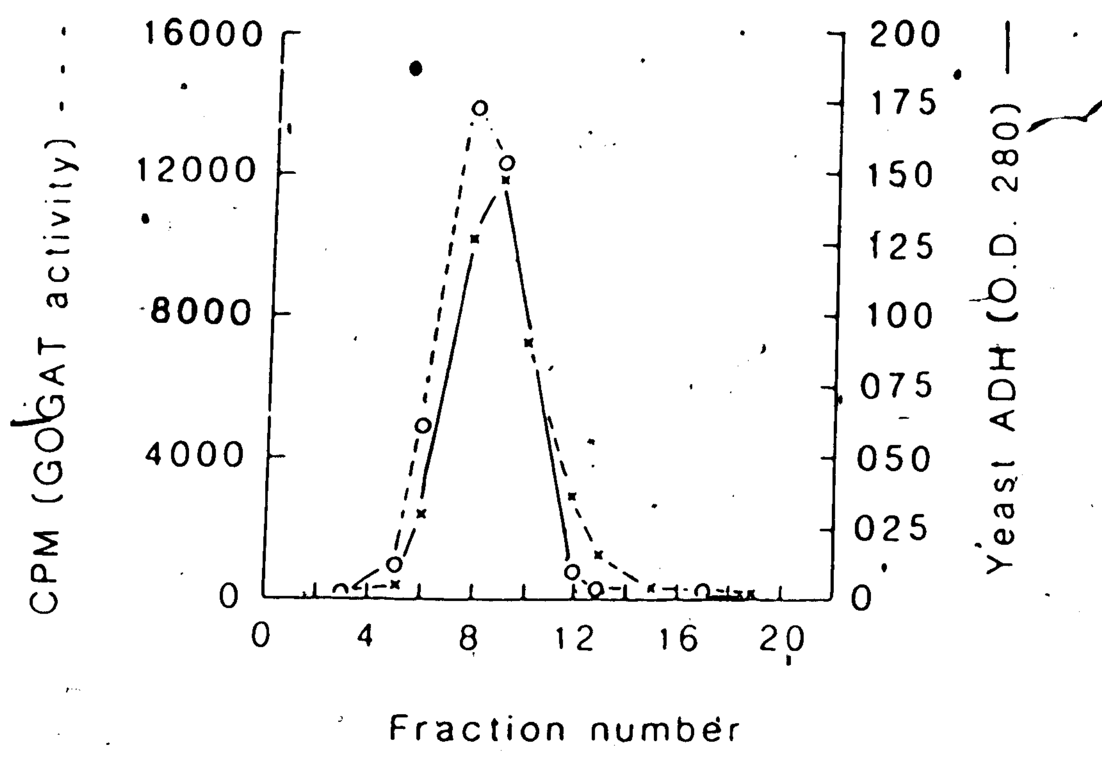


Figure 8 A plot representing the distribution of fd-dependent GOGAT activity and the absorbancy of yeast ADH collected from separate sucrose gradients. This experiment was repeated once, with similar results.

1982) in V. faba as a monomer (Wallsgrove et al. 1977).

Reciprocal plots are presented in Figures 9 and 10. The enzymatic reaction resembles a sequential mechanism, with a Km for 2-oxoglutarate of 100 uM and for glutamine of 380 uM. The plots represent data originating from one preparation of fd-dependent GOGAT. The Km for ferredoxin was found to be 1.2 to 2 uM (Fig. 11). These values are similar to other reported fd-dependent GOGAT proteins (Table 6).

The fd-dependent GOGAT activity in roots of B. napus was partially characterised once. The procedure for isolation followed that of the leaf fd-dependent GOGAT. The fd-dependent GOGAT activity behaved similarly as the fd-dependent GOGAT from the leaf form, but differently than NADH-dependent GOGAT (when chromatographed on a DEAE-cellulose preparative column, Fig. 12). However, not enough enzyme was recovered to justify further purification.

Genetic Analysis of GLUS Mutants of A. thaliania

In order to test for the number of genetic complementation groups, sexual crosses among the mutants were made. All the mutants were crossed individually to each of the following three maternal lines: CS 5, CS 66, CS 254. These three maternal lines were marked with the glaborous phenotype, in order to differentiate self and out-crossed progeny. In all cases the progeny (593 individuals) were inviable in normal air. Sixty-two progeny were selected at

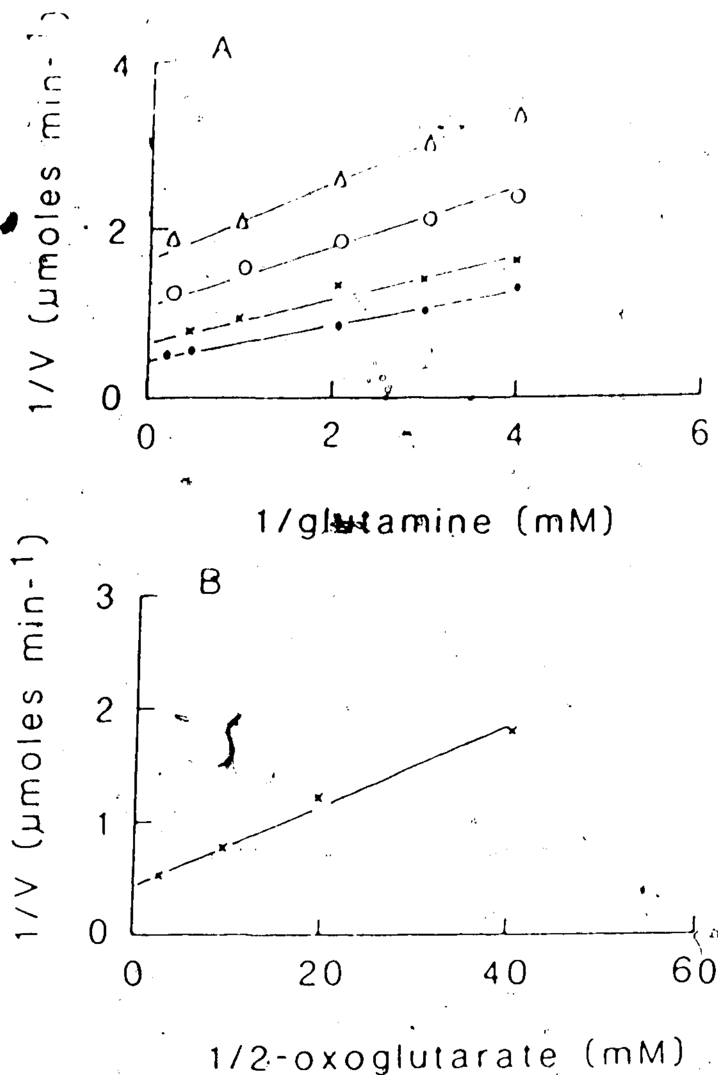


Figure 9 A. Plot of 1/v versus 1/[glutamine] at fixed concentration of 2-oxoglutarate for fd-dependent GOGAT. The 2-oxoglutarate concentrations were: ● =500 uM; x =100 uM; o =50 uM; Δ =25 uM. B. Plot of intercepts of plots of 1/v versus 1/[glutamine] at fixed concentrations of 2-oxoglutarate versus 1/[2-oxoglutarate].

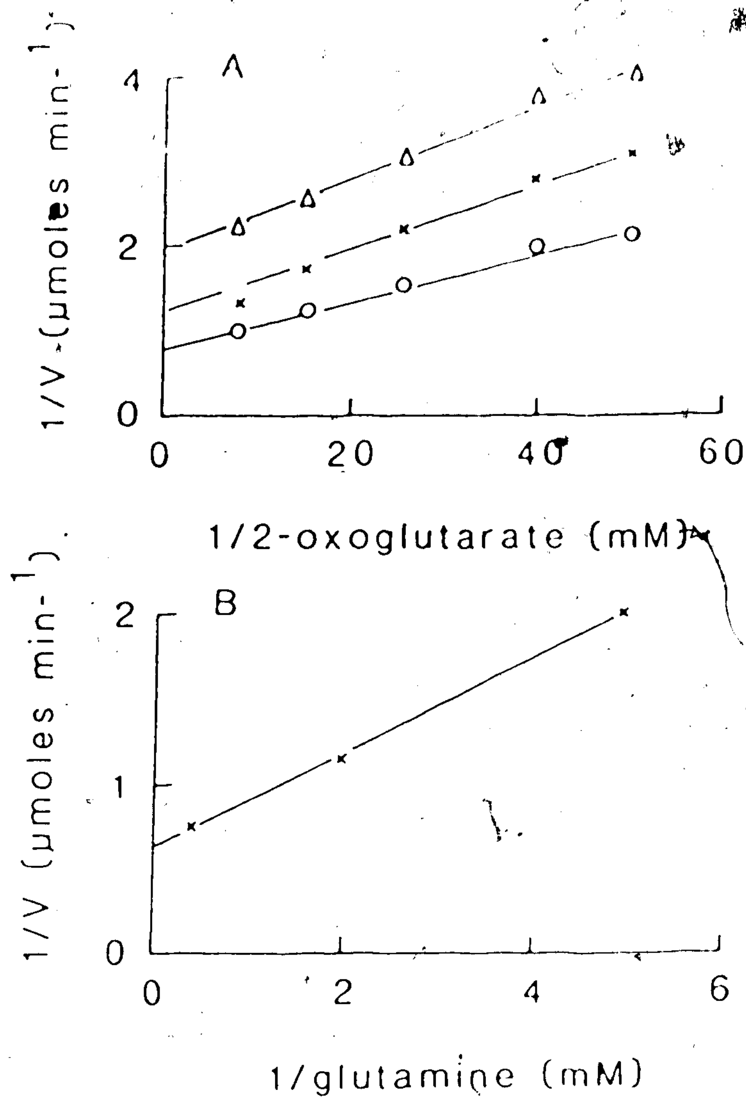


Figure 10 A. Plot of 1/v **versus** 1/[2-oxoglutarate] at fixed concentrations of glutamine for fd-dependent GOGAT. The glutamine concentrations were: Δ =0.2 mM; x 0.5 mM; o =2.5 mM. B. Plot of intercepts of plots of 1/v versus 1/[2-oxoglutarate] at fixed concentrations of glutamine versus 1/[glutamine].

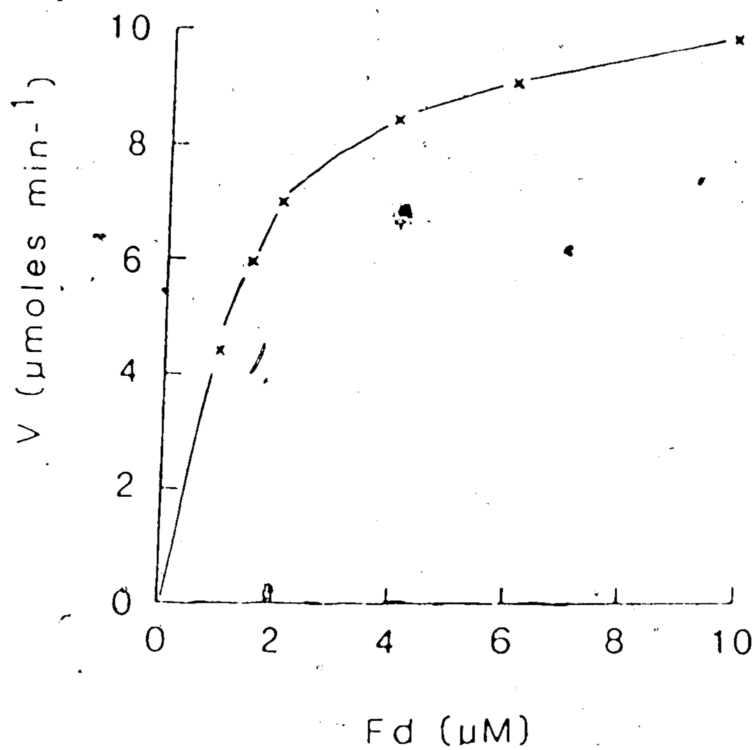


Figure 11 Plot of initial leaf fd-dependent GOGAT activity versus varying ferredoxin concentration.

Table 6

Properties of NADH and fd GOGAT preparations reported in the literature. N.A. indicates information not available.

Source	K_m (µm)				Specif. Act. (U/mg)	Subunit MW	Ref.
	fd	NADH	2-OXG	GLN			
Lupin	-	1.3	39	400	18.0	225,000	Boland, Benny
Vicia	2	-	150	330	7.0	145,000	Wallsgrove et al. 1983
Corn	1.7	-	240	1100	14.1	160,000	Matoh et al 1979
Pea	-	4.0	37	400	15.6	220,000	Matoh et al 1980
Chlamydomonas	0.01	-	170	190	N.A.	165,000	Cullimore, Sims 1981
Chlamydomonas	-	13.0	7.0	900	N.A.	240,000	Cullimore, Sims 1981
Rice	5.5	-	330	270-570	36.0	115,000	Suzuki, Gadal 1982
Chlamydomonas	5.5	-	1000	700	10.0	146,500	Galvan et al. 1984
Chlamydomonas	-	7.6	18.2	600	4.6	N.A.	Marquez et al. 1984
B.napus	-	4.5	80	590	21.2	225,000	thesis
B.napus	-	4.0	80	625	20.0	227,000	thesis
B.napus	1.2	-	100	380	42.1	150,000	thesis

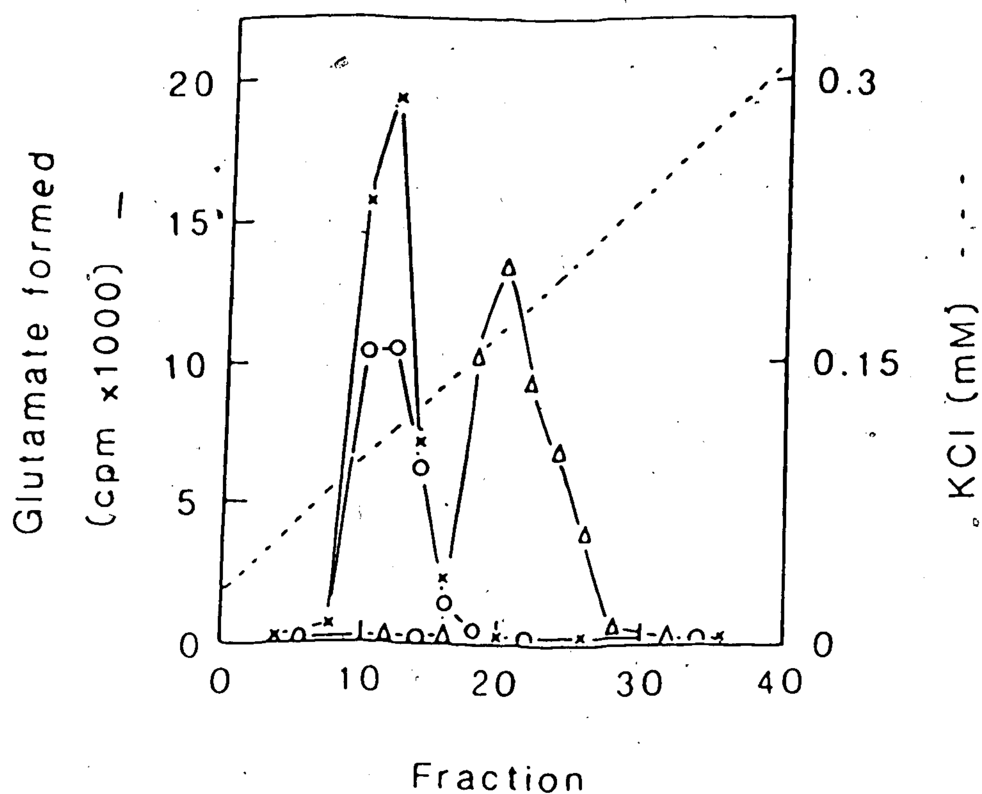


Figure 12 Chromatographic patterns of GOGAT activity on DEAE-cellulose preparative columns. GOGAT activities were assayed for fd-dependent activity from the leaf (x), the root (o) and for NADH-dependent activity from the leaf (Δ). The data are taken from three different DEAE-cellulose columns.

random from amongst all three crosses and were found to be deficient in fd-dependent GOGAT activity (not shown). In crosses of each mutant to the WT strain, all F_1 progeny were viable, demonstrating the recessive nature of the mutation. Therefore, it was concluded that the same gene was responsible for the alteration in fd-dependent GOGAT in these mutants.

Assay for GOGAT Activities in GLUS Plants

The metabolic distribution of products of $^{14}CO_2$ fixation found in GLUS strains photosynthesizing in normal air suggested a defect for fd-dependent GOGAT activity (data of Dr. C. R. Somerville, pers. comm.) and thus crude extracts were prepared from plants grown in CO_2-enriched air and assayed for fd-dependent GOGAT activity in root and leaf tissue. As shown in Table 7, the activity in the mutants was reduced in comparison to the amounts determined in the WT strain. In contrast, the NADH-dependent GOGAT activity was normal in both root and leaf tissue. It is conceivable that the lack of enzyme activity found in vitro in some or all of the mutants, was due to enzymatic instability during the isolation procedure. This could be the result of structural alterations leading to osmotic instability, or an increased susceptibility to protease activity. Therefore, the assays were repeated in the presence of 30% glycerol or the protease inhibitor, PMSF whilst grinding the tissues. Under these conditions, increases of 2-11% in fd-dependent GOGAT

Table 7

GOGAT activities and CRM levels for GLUS mutants of **A. thaliania**

Strain	GOGAT activity				CRM level
	leaf		root		
	fd	NADH	fd	NADH	(%)
	(µmoles glutamate mg $protein^{-1}$ hr^{-1})				
WT	5980	296	280	588	100
CS 5	180	241	64	480	23
CS 6	220	280	70	380	69
CS 30	228	298	102	460	48
CS 37	210	340	84	580	72
CS 39	160	350	64	560	31
CS 40	250	345	70	490	26
CS 56	160	293	65	500	40
CS 66	200	334	71	450	60
CS 102	195	245	77	540	56
CS 103	230	305	62	480	27
CS 105	155	335	55	600	16
CS 110	200	345	70	498	45
CS 112	250	372	64	536	42
CS 113	235	350	60	560	8
CS 138	150	390	62	582	7
CS 157	152	380	65	555	29
CS 162	253	340	65	490	10
CS 168	161	275	66	395	53
CS 172	205	365	64	564	18
CS 204	250	390	66	597	30
CS 206	235	344	64	505	11
CS 239	210	370	69	443	28
CS 254	145	330	60	509	8
CS 260	170	350	74	507	18
CS 261	250	380	78	490	44
CS 263	210	370	74	480	45

activity were found (not shown). The use of glycerol and PMSF has been reported to stabilise mutationally altered proteins in **Saccharomyces cerevisiae** (Bigelis et al. 1981).

Immunological Analyses of GLUS Plants

The possibility that the mutants possessed altered forms of fd-dependent GOGAT was investigated immunologically. An antibody was prepared by injecting rabbits with fd-dependent GOGAT purified from spinach. It was found that the antibody specifically inhibited fd-dependent GOGAT activity from **A. thaliania** leaf tissue, but did not inhibit NADH-dependent GOGAT (Fig. 13). The level of CRM present in each mutant was assayed by the ability of mutant extracts to protect WT fd-dependent GOGAT activity from the antibody. A range of CRM values from 7 to 72% was found in the mutants (Table 7). These data provide support to the notion that the protein has been mutationally altered.

Antibody-antigen complexes precipitated from leaf extracts treated with GOGAT antibody using **S. aureus** absorbent, were washed, resuspended in SDS and subjected to SDS-PAGE. Plates 3 and 4, show the immunoprecipitates from 25 mutant strains of **A. thaliania**. As shown, three bands appear per lane of the gel. The bottom band is IgG, the other two bands are either fd-dependent GOGAT polypeptides or the middle band represents another protein. The top one migrates according to the expected MW of the protein.

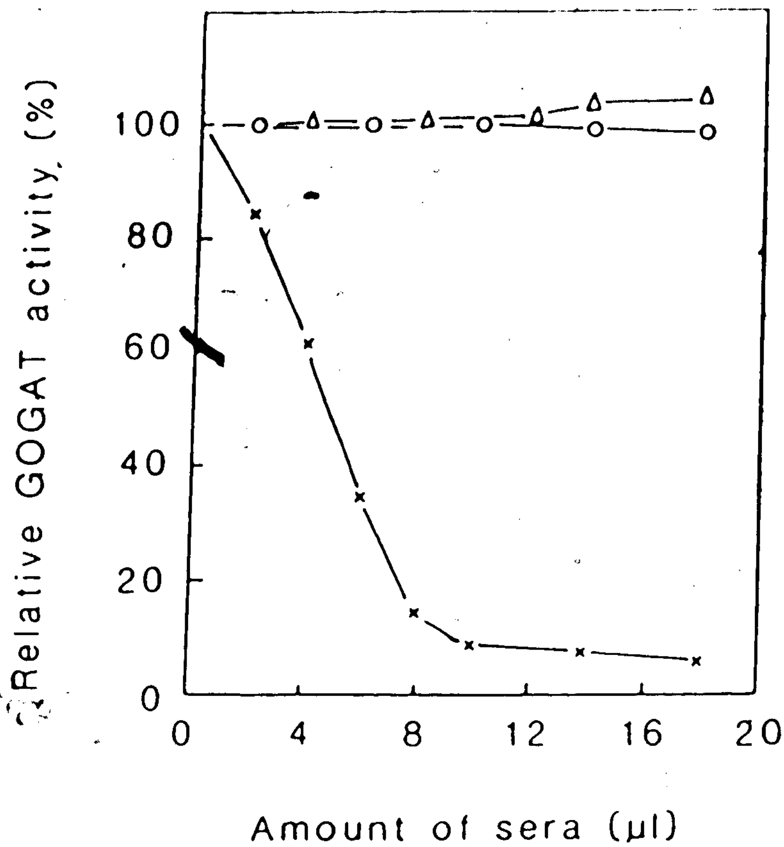
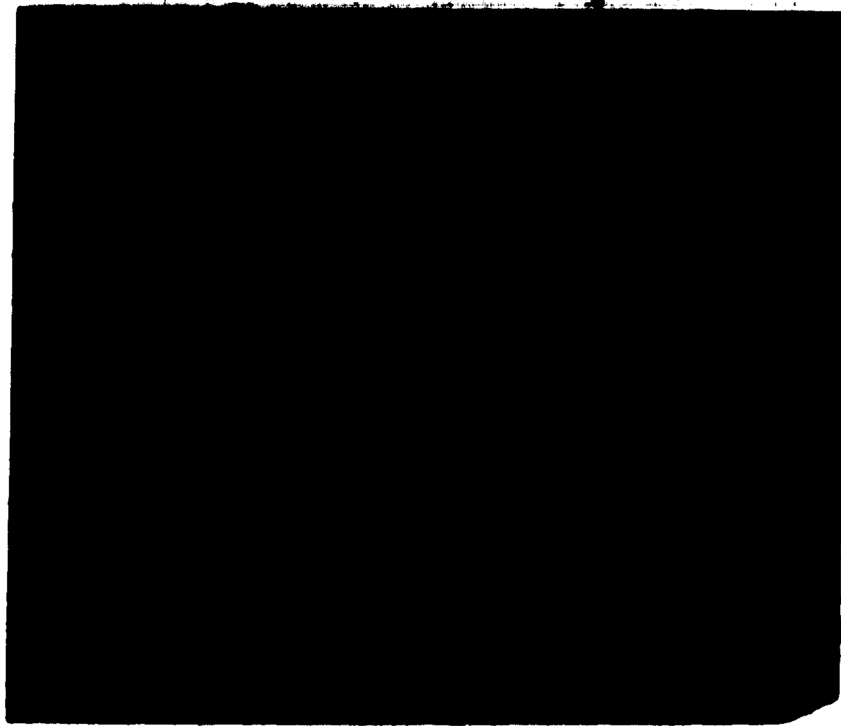


Figure 13 The immunoinhibition of NADH- and fd-dependent GOGAT from leaves of **A. thaliania.** The data shown were obtained using crude sera. Fd-dependent GOGAT activity after treatment with control sera (o) and antisera (x). NADH-dependent GOGAT activity after treatment with antisera (Δ).

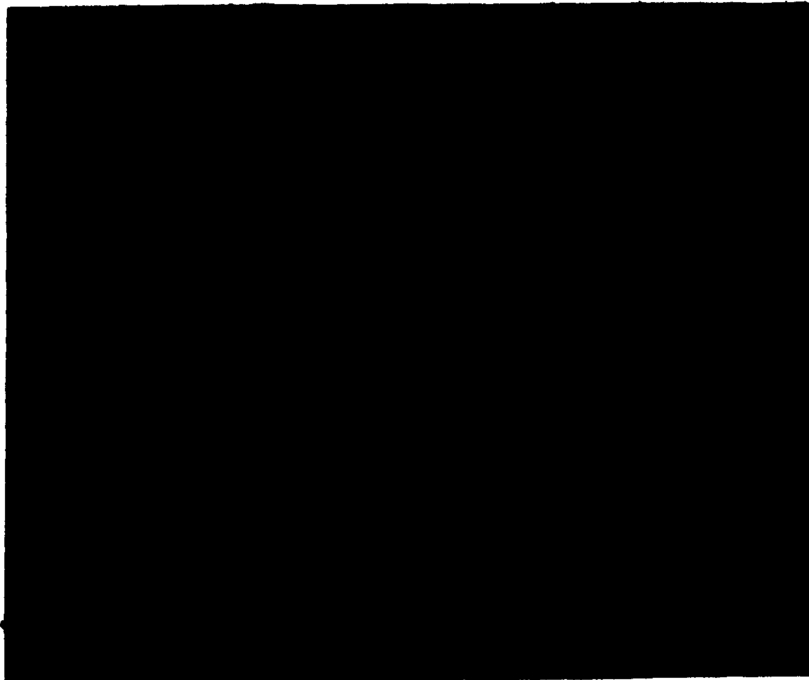
Plate 3 A 7.5% SDS-PAGE of proteins removed from *A. thaliania* crude extracts by antibody raised against spinach leaf fd-dependent GOGAT. Migration of polypeptides is from top to bottom. The "S" lane contains MW markers, which are (from top to bottom) myosin (200,000), β-galactosidase (118,000), phosphorylase B (92,000), BSA (67,000) and ovalbumin (45,000). For comparison, WT is the wild-type strain. The gel was stained with Coomassie blue. The number of the appropriate **GLUS** strain or WT is indicated on the lanes of the gel. Gels A and B were identical but contained immunoprecipitates from different strains.

A



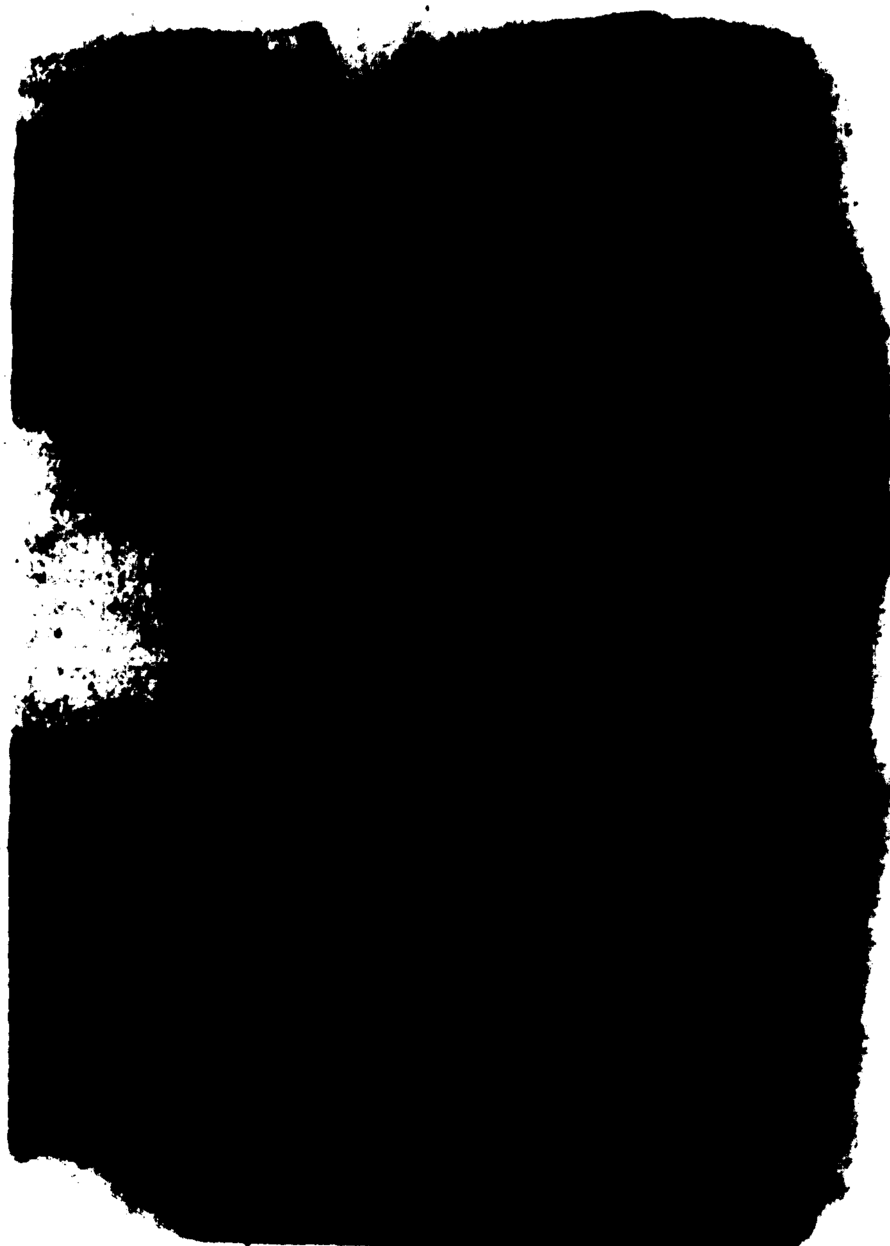
105wt 6 S 138 40 254 56 103

B



168 105 261 5 39 254 S 263

Plate 4 SDS-PAGE of proteins removed from A. thaliania crude extracts by antibody raised against spinach leaf fd-dependent GOGAT. Except for different strains used the gel is similar to the one reproduced in Plate 3.


37
159
wt
157
140
30
168
206
162
172
112
66

Initially, immunoprecipitates were allowed to form overnight, and then incubated with S. aureus absorbent for 1 hr at 4° C. Later it was determined that a complex could be formed in 3-4 hr and treated with S. aureus absorbent for 10 min. With the alternative procedure, involving the shorter incubation periods, immunoprecipitated polypeptides were resolved on a 7.5% SDS-PAGE, and only the fd-dependent GOGAT polypeptide was apparent above the IgG band. The middle band was no longer present (Plate 5). In lanes C and D are WT and CS-162 strain precipitates, showing identical migration for fd-dependent GOGAT, and no middle band. In lanes A and B, are the precipitates for the WT and CS-162 strains, using the original protocol. The lower MW polypeptides in lanes C and E are of unknown origin.

In Plate 6 are total soluble polypeptides prepared from leaves resolved by SDS-PAGE on 7.5% gels. Seventeen mutant strains were examined. In the A gel, an immunoprecipitate was prepared and resolved on the gel. As shown, a band corresponding to the fd-dependent GOGAT peptide is apparent in the total polypeptides from the WT strain. As surveyed with leaf polypeptides extracts or immunoprecipitates, using SDS-PAGE, no mutant strain was found to have a fd-dependent GOGAT altered in MW (Plate 6).

One possible explanation for the low fd-dependent GOGAT activity and CRM level in the mutants, would be that the protein had been mutationally altered, to such an effect

Plate 5. A comparison of proteins immunoprecipitated by two procedures, using the same antibody to spinach fd-dependent GOGAT. Migration of proteins is from top to bottom. Lanes A and B contain polypeptides prepared by the one procedure and lanes C and E by the alternative procedure (see Materials and Methods). Lanes A and C contain immunoprecipitates from WT tissue and lanes B and E from CS 162. Lane D contains the MW markers phosphorylase B, BSA and ovalbumin. The gel was stained with Coomassie blue.

– fd GOGAT
– IgG
A B C D E

Plate 6 A. 7.5% SDS-PAGE of total soluble polypeptides from leaves of **A. thaliania**, WT and **GLUS** strains. Migration is from top to bottom. In gel A, immunoprecipitated polypeptides were also subjected to electrophoresis (lane "IM"). The top band in the IM lane is fd -dependent GOGAT. The gels were stained with Coomassie blue.

A

B

C

that it was no longer in the proper environment. It is believed that fd-dependent GOGAT is synthesised in the cytoplasm (Suzuki and Gadal 1984), and transported into the chloroplast. To investigate whether or not the improper localisation of fd-dependent GOGAT (within the cell) was the cause for the mutant phenotype, chloroplasts were isolated and mixed 1:4 (vol/vol) in the I-grind buffer. Chloroplasts from both CS 30 and CS 66 contain fd-dependent GOGAT polypeptide (Plate 7). These two high CRM mutants were used, as the yield of chloroplasts is sometimes low in the mutant strains. For example when using CS 254, little success was achieved in immunoprecipitating fd-dependent GOGAT from the chloroplast preparations, but a faint band was sometimes apparent (not shown). It was concluded that a more resolving protocol is required for the determination of the localisation of fd-dependent GOGAT polypeptide within the chloroplasts of strain CS 254. It is the author's view that a protocol utilising radiolabeling of the proteins might yield a greater resolving power.

The lowered activity for both root and leaf fd-dependent GOGAT activity may be related to the same gene defect. An immunological approach to answer this question was attempted. The antibody raised against spinach leaf fd-dependent GOGAT, did immunoprecipitate a protein from the roots of the WT strain. The protein migrated to a position equivalent to the position of purified spinach fd-dependent

Plate 7 Fd-dependent GOGAT polypeptide immuno-precipitated from isolated intact chloroplasts, with antibody against spinach leaf fd-dependent GOGAT. Migration is from top to bottom. Lane A contains polypeptides from the mutant strain, CS-30 and lane B contains polypeptides from CS-66.

– fd GOGAT

– IgG

A B

GOGAT did (Plate 8). The apparent antigenic similarity would suggests that leaf and root fd-dependent GOGAT are possibly encoded by the same gene. Also, it suggests that a gene thats expression is affected by light (Suzuki and Gadal 1984) can be expressed in the root tissue.

Photosynthetic CO_2 Exchange Rates of WT and GLUS Plants

In Figure 14 the net exchange of CO_2 is presented for both strains. In 2% O_2 and 360 ppm CO_2, (remaining N_2) the rates were similar. In 21% O_2 and 349 ppm CO_2 (remaining N_2), **GLUS** photosynthesis was inhibited after 5 to 8 min from the onset of illumination. This response to a photorespiratory gas regime has been observed previously with the **GLUS** strain (Somerville and Ogren 1980a) and was typical for a photorespiratory-defective mutant (Somerville and Ogren 1982a).

Activity of Key PCR Cycle Enzymes in WT and GLUS Plants

Light induces the activation of RuBISCO in **A. thaliania** (Somerville et al. 1982) and as shown in Figure 15, RuBISCO was similarly activated in the WT and **GLUS** strain under photorespiratory gas conditions. An approximate doubling of enzyme activity (when compared to the enzyme activity of an extract prepared from leaves) was observed when exposed to light and equilibrated for 20 min in the dark in the IRGA. However, this exposure of **GLUS** plants to 21% O_2 and 340 ppm CO_2 (remaining N_2) eventually resulted in reduced RuBISCO

Plate 8 A 7.5% SDS-PAGE of purified spinach leaf fd-dependent GOGAT (lane A) and polypeptides removed from **A. thaliania** root tissue (lane B) by antibody raised against the leaf fd-dependent GOGAT. Migration is from top to bottom. The gel was stained with Coomassie blue.

- fd GOGAT
- IgG

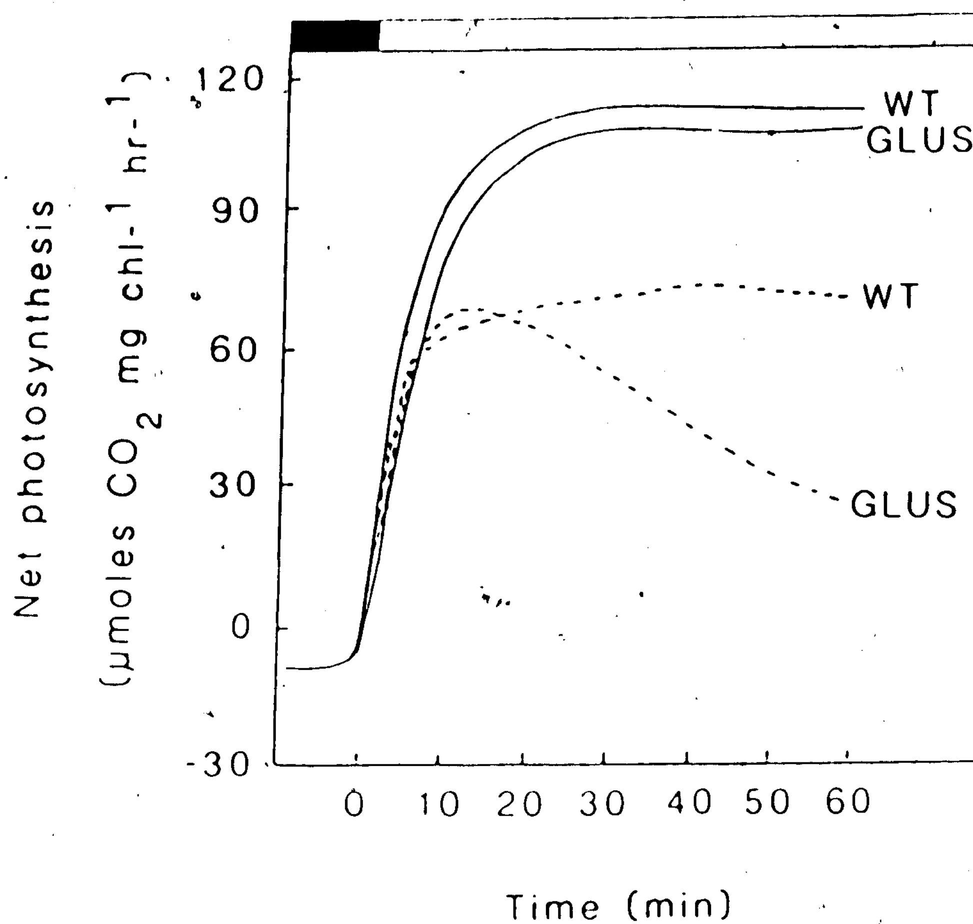


Figure 14 Rates of net photosynthesis in **WT** and **GLUS** mutant **A. thaliania** illuminated under 2% O_2 and 360 ppm CO_2 (remaining N_2) (----) or 21% O_2 and 349 ppm CO_2, (remaining N_2) (- - -). The photosynthesis data were collected from 5 to 7 plants for each strain. Plants were 28 to 35 days old, and of two different seeding dates.

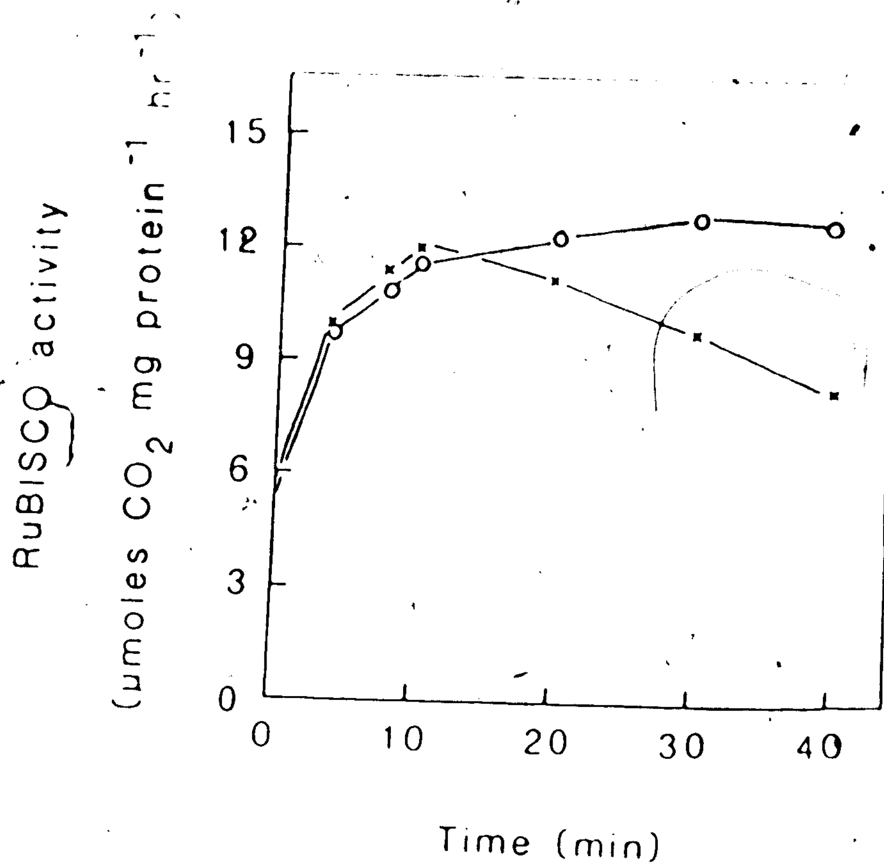


Figure 15 The effect of 21% O_2 and 349 ppm CO_2, remaining N_2 on the RuBISCO activity from leaves of WT (**O**) and **GLUS** mutants (**x**) of **A. thaliania**. Plants were allowed to photosynthesize for the times shown, then were immersed in liquid N_2 and the leaves removed under liquid N_2. RuBISCO was extracted and assayed as described in the text. The plants were 27 to 35 days old and each datum point is the average of 10 to 14 plants.

activity. After 40 min of photosynthesis, the RuBISCO activity in the GLUS strain was less than 70% of the WT RuBISCO activity.

Two other enzymes of the PCR cycle, known to be light-activated (Lilley 1983) were also assayed. As shown in Tables 8 and 9, FBPase and R5P kinase activities were lower in the GLUS plant then in the WT under photorespiratory gas conditions. After 40 min of photosynthesis, FBPase activity in the GLUS was 76% of the WT level, whereas the R5P kinase activity was 69% of the WT level when performed under similar conditions.

Enzymatic Analysis of RuBP in WT and GLUS Plants

Previously, it had been proposed that the decline in CO_2 fixation for GLUS plants was the result of a lack of carbon in the PCR cycle (Somerville and Ogren 1983). To examine this proposal, RuBP levels were enzymatically determined in both the WT and mutant strain. The RuBP level was analysed per mg protein, as this was thought to be the most accurate method with $HClO_4$ extracts. When chlorophyll and protein were determined with leaf extracts prepared in RuBISCO-buffer, approximately 10 mg of soluble protein was equivalent to 0.7 mg chlorophyll. Thus, a steady-state RuBP level of 10.2 nmol mg protein^{-1} (Table 10) is equal to 146 nmol mg chlorophyll^{-1}. This assumes that an equivalent amount of protein was extracted by grinding in RuBISCO-

Table 8

The FBPase activity from leaves of plants that photosynthesized under nonphotorespiratory conditions (2% O_2 and 360 ppm CO_2, remaining N_2) and during photorespiration (21% O_2 and 349 ppm CO_2, remaining N_2). Plants were 30 to 35 days old. This experiment was done with plants from one seeding date. The exp[illegible]ment was repeated twice for the 21% O_2 regime and [illegible]rformed only once for the 2% O_2 regime. For the 2[illegible] O_2 determination, the results were averaged (± [illegible]

Time of illumination (min)	FBPase activity (μmoles mg chl^{-1} hr^{-1})			
	2% O_2		21% O_2	
	WT	GLUS	WT	GLUS
0	41.3	37.0	37.6±2.1	33.5±1.9
5	-[1]	-	77.1±3.0	76.0±3.2
10	105.3	96.8	92.3±3.6	81.6±3.7
20	-	-	93.4±3.4	78.7±3.5
40	101.9	90.6	90.8±3.2	69.3±3.2

-1 data not collected.

Table 9

R5P kinase activity from leaves photosynthesizing as in Table 3.3. The 2% O_2 experiment was carried out once. The 50% O_2 experiment was repeated three times and from plants of two different seeding dates. The plants were 29 to 36 days old. The 50% O_2 data were averaged (± S.E.).

Time	R5P kinase			
(min)	2% O_2		50% O_2	
	WT	**GLUS**	**WT**	**GLUS**
	(μmoles mg chl^{-1} hr^{-1})			
0	63.0	71.8	69.3±4.4	74.5±4.7
10	323.9	321.1	386.7±15.5	352.8±20.4
20	309.2	348.9	402.0±16.1	337.0±19.3
40	346.9	328.4	390.3±21.7	270.4±15.9

Table 10

Change in RuBP levels during photosynthesis in 21% O_2 and 349 ppm CO_2 (remaining N_2) for **GLUS** and WT **Arabidopsis**. The values are from 4 separate experiments and were averaged (± S.E.).

Time(min)	RuBP (nmoles / mg protein) WT	**GLUS**
4	13.5±2.9	12.8±2.4
8	12.4±2.6	13.0±2.5
16	11.1±2.0	9.9±2.8
45	10.2±2.0	4.7±1.7

buffer as was extracted for $HClO_4$ grinds. This value is comparable to the RuBP level as reported in other studies (Creach and Stewart 1982; Perchorowizc and Jensen 1983; Dietz and Heber 1984a; Badger et al. 1984). The reliability of the procedure used to measure RuBP, was assessed by recovery measurements for duplicate samples of RuBP. Both duplicates were added directly to $HClO_4$, although one was combined with ground leaf tissue stored in $HClO_4$. By comparing the RuBP values of the two samples, an estimated recovery of 83.4 $\pm$ 12.0 (average of 5 trials $\pm$ S.E.) was noted. (Values presented in Table 10 are corrected for yield loss.) However, this may not be an accurate way to estimate RuBP stability in the tissue. RuBP added to $HClO_4$ was assumed to be stable, however, the RuBP in the tissue could be metabolised and / or degraded during the process of killing the tissue, as liquid N_2 does take time to penetrate leaf tissue (Ap Rees 1974). Therefore, the RuBP levels reported herein may not accurately measure the absolute *in vivo* level, but are probably valid for comparing RuBP levels between strains.

As shown in Table 10, after 4 to 8 min of photosynthesis in 21% O_2 and 349 ppm CO_2 (remaining N_2), the GLUS strain's RuBP level was 95% of that of the WT strain. By 8 min, the RuBP level was 104% and by 45 min, the level was 43%. These data suggest that RuBP may limit the rate of photosynthesis between 16 and 45 min. However, no difference in RuBP levels of mature leaves between the GLUS and WT was

found after 4 to 8 min in photorespiratory conditions, when inhibition of photosynthesis was first observed in the **GLUS** plants (Fig. 14).

The Effects of NH_4^+ on A. thalian[illegible] [illegible]siology

The previous study concerning RuBP levels showed that a difference in levels was present between the **GLUS** and WT plants. The lower level of **RuBP** after 45 min could be the result of accumulated NH_4^+ which in turn had an uncoupling effect on photophosphorylation. To test the above, NH_4Cl was f[illegible]lants which had their roots severed, 1 to 3 mm from [illegible]oot tip. Previously, the leaf-fragment technique for [illegible]ing NH_4Cl was used in **A. thaliania** (Somerville and [illegible]erville 1983), yielding unusual $^{14}CO_2$ labeling patterns (Somerville and Ogren 1981). Therefore, and in order to compare metabolism with intact plants, the severed root tip technique was used.

WT plants with severed roots still had a photosynthetic CO_2 exchange rate similar to that of intact WT plants (Fig. 16). The observed photosynthetic rates of about 60 µmole CO_2 mg chl^{-1} hr^{-1} in 21% O_2 and 334 ppm CO_2 (remaining N_2) are comparable to other studies with **A. thaliania** (McCourt 1983; Somerville and Somerville 1983). After 30 min, the CO_2 exchange rate was inhibited by over 50% in the **GLUS** strain relative to the WT. The data indicate (Fig. 16) that that feeding NH_4Cl to WT plants of **A. thaliania** was inhibitory to

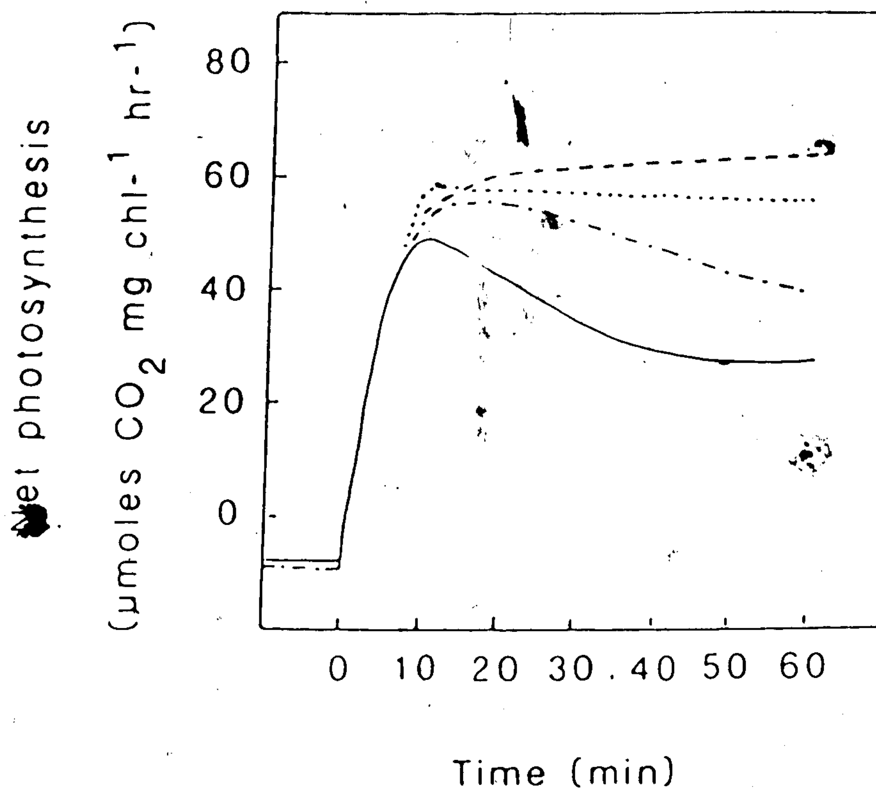


Figure 16 Net photosynthetic rate for WT (- -), GLUS (——), control WT with cut roots (····) and NH_4Cl-fed WT (- · -) plants in a gas regime of 21% O_2 and 334 ppm CO_2 (remaining N_2).

photosynthesis. (Data will be presented later showing that NH_4Cl also inhibits photosynthesis in chloroplasts).

Figure 17 shows the NH_4^+ levels determined in plants under the same conditions as quoted in Figure 16. In WT plants, little NH_4^+ was detected. This indicated an active glutamine synthetase/GOGAT NH_4^+ assimilatory cycle (Keys et al. 1978). In 21% O_2 and 334 ppm CO_2 (remaining N_2), the relationship between NH_4^+ accumulation and photosynthetic rate in the **GLUS** strain was not as clear as reported for 50% O_2 (Somerville and Ogren 1980a). Between 5 to 10 min after the onset of illumination a slight decline in photosynthesis in **GLUS** plants could be detected, yet, the NH_4^+ level did not seem appreciably higher than in the WT plant.

Upon illumination, the NH_4^+ level rose quite rapidly in NH_4Cl-fed WT plants, and reached higher levels than found in either the WT control or the **GLUS** strain (Fig. 17). Probably, the higher NH_4^+ levels were in response to transpiration. Despite the higher NH_4^+ level of the NH_4Cl-fed plants, photosynthesis was not inhibited to the same degree as the **GLUS** plant. For instance, after 20 min of illumination, when the NH_4^+ level had reached approximately 0.24 µmole mg chl^{-1}, the **GLUS** photosynthetic rate had decreased by 40%. However, in the NH_4Cl-fed WT plant NH_4^+ had reached approximately 0.26 µmoles mg chl^{-1} after 20 min of illumination and photosynthesis was not inhibited. Clearly, if the build-up, of NH_4^+ was the sole cause for the inhibition of photosynthesis in **GLUS** plants, then an

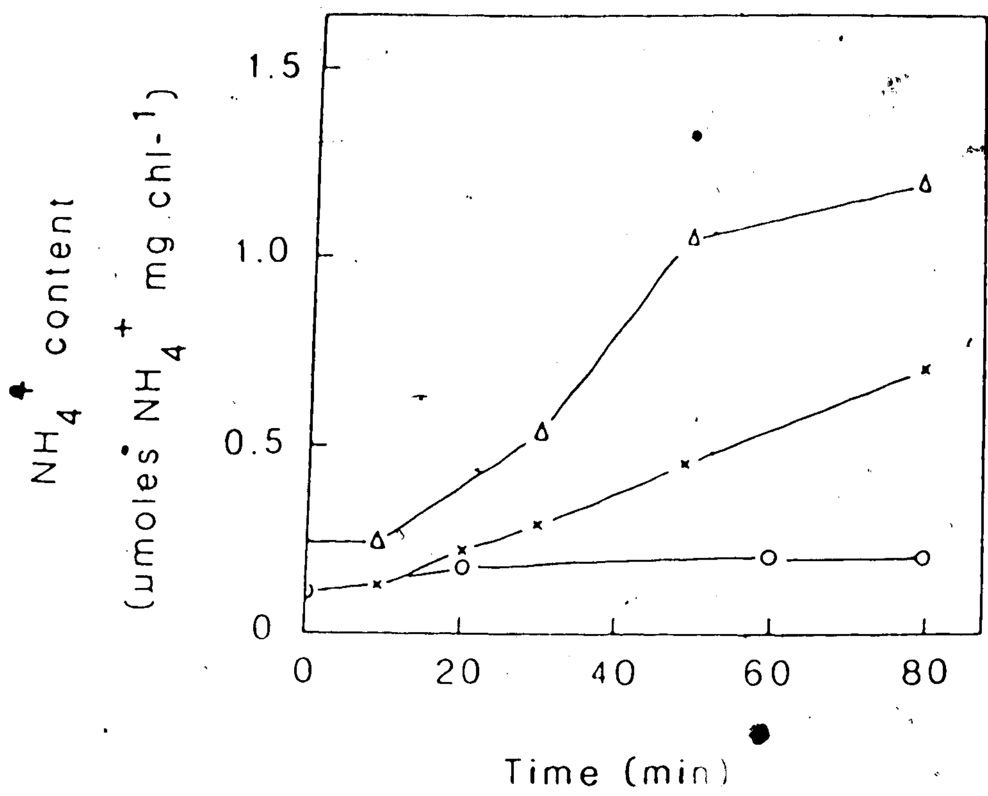


Figure 17 The accumulation of NH_4^+ in **WT** (o), **GLUS** (x) and NH_4Cl-fed WT (Δ) plants of *A. thaliania*. The gas regime was 21% O_2 and 34 ppm CO_2 (remaining N_2).

equivalent inhibition of photosynthesis should be observed in the NH_4Cl-fed plants. Thus, a separate or additional mechanism may be responsible for photosynthetic inhibition in GLUS plants.

To understand the consequences of the GLUS lesion on metabolism in a leaf undergoing photorespiration, the metabolisms of the GLUS and WT strain were compared by measuring the percent recovery of ^{14}C in photosynthetic intermediates following labeling of the plants with $^{14}CO_2$ (Table 11). After labeling the plants for 2.5 min beginning 4 min after the onset of illumination, the percent recovery of ^{14}C metabolites was similar in the two strains. However, if the labeling was done in the 11-13.5 min interval after illumination, a higher percentage of label was present in SMP, glycolate, glyoxylate, 2-oxoglutarate and RuBP in the GLUS strain, than in the WT. Some of these changes were probably the result of the lack of glutamate for transamination of glyoxylate to glycine. Hence, ^{14}C accumulated in glyoxylate to 1.9% of the total ^{14}C recovered from the GLUS strain and less ^{14}C was recovered in serine and glycine. The cause(s) of other alterations were not as obvious. At the time of the experiment, the percent accumulation of ^{14}C in RuBP was unexpected.

In 1982, Cook and Tolbert reported that glyoxylate can directly inhibit RuBISCO and their finding stimulated a brief investigation into the level of glyoxylate in GLUS

Table 11

Percentage of total radioactivity recovered in intermediates isolated from **GLUS** and WT leaves, which photosynthesized in 21% O_2 and 334 ppm CO_2. $^{14}CO_2$ fixation was measured during the intervals after the onset of photosynthesis: 4 to 6.5 and 11 to 13.5 min. Plants were allowed to equilibrate with the gas system of the IRGA in the dark for 20 min before illumination. This experiment was replicated three times (average ± S.E.).

Fraction	Strain			
	WT		**GLUS**	
	4-6.5	11-13.5	4-6.5	11-13.5
Acid-1	7.6	10.3	8.1	14.3
malate	0.4±0.1	3.0±0.6	1.8±0.4	4.9±0.6
citrate	0.0±0.0	0.3±0.1	0.0±0.0	0.6±0.1
2-oxo-glutarate	0.0±0.0	0.0±0.0	0.2±0.1	0.8±0.2
glycolate	1.2±0.1	0.3±0.1	1.9±0.3	2.2±0.2
glycerate	1.2±0.1	2.0±0.2	1.8±0.2	1.0±0.2
glyoxylate	0.0±0.0	0.0±0.0	0.5±0.1	1.9±0.2
Acid-2	40.1	31.6	35.7	39.2
PGA	19.9±1.4	14.7±1.0	15.9±1.5	10.4±1.4
SMP	15.4±2.1	12.0±2.3	14.1±2.4	24.1±2.5
Acid-3	17.0	14.6	19.0	19.3
RuBP	10.5±1.3	8.7±0.9	14.0±1.1	13.1±1.2
FBP	3.2±0.4	2.6±0.5	3.9±0.5	4.1±0.4
Basic	19.3	24.8	20.9	16.5
glycine	11.2±1.1	12.4±1.0	12.1±0.6	8.9±0.8
serine	5.5±0.7	7.9±0.8	7.4±0.9	5.3±0.5
glutamate	0.9±0.2	2.0±0.4	0.8±0.2	0.8±0.2
Neutral	7.9±0.5	12.6±1.0	8.8±0.4	6.9±0.7
Insoluble	1.8±0.1	4.9±0.4	1.4±0.1	3.3±0.4
Recovery (%)	93.7	98.8	93.9	99.2
DPM recovered (10^6)	1.79	3.12	1.63	1.92

plants. In the $^{14}CO_2$ labeling experiment described in Table 11, ^{14}C-glyoxylate accumulated in the GLUS strain. Therefore, the relationship between glyoxylate metabolism and photosynthesis was examined further. Plants were allowed to photosynthesize for 30 min in 50% O_2 and 335 ppm CO_2 (remaining N_2) at which time $^{14}CO_2$ was introduced. Photosynthesis was continued for the next 20 min in the presence of $^{14}CO_2$. It had previously been shown that photorespiratory-inhibited plants placed in the dark will restore their initial photosynthetic rate, by an unknown means (Somerville and Ogren 1982c). Here, after the 20 min labeling period, plants were flushed with normal air in the dark. If glyoxylate was responsible for the inhibition of photosynthesis in GLUS plants then the rate of restoration of photosynthesis in the GLUS plants should correspond to the rate of glyoxylate disappearance. However, this was not the case. After 20 min of $^{14}CO_2$ fixation, 4.8% (82,752 dpm) of the total radioactive carbon recovered was in glyoxylate. After 15 min of darkness (the first time point examined) the ^{14}C in glyoxylate had disappeared. Yet, under the particular conditions used, the GLUS plants had not restored their initial rate of photosynthesis. In fact, full restoration required 40-45 min (not shown, but a similar curve is presented in Fig. 18; see also Somerville and Ogren 1982c). Therefore, it was concluded that some other factor besides glyoxylate was responsible for the lengthy restoration period of GLUS plants. Because glyoxylate might be quite

unstable during extraction from the plant tissue, this experiment should be repeated with 2,4-dinitrophenylhdrazine treated extracts (Hatch and Slack 1966) to allow for a more accurate glyoxylate determination.

To determine what metabolites were altered after long term photosynthesis in **GLUS** plants and NH_4Cl-fed WT plants, $^{14}CO_2$ was supplied for 10 min to plants which had photosynthesized for 50 min in 50% O_2 and 335 ppm CO_2 (remaining N_2). This gas regime was used, instead of a 21% O_2 one, in order to have a faster inhibition of photosynthesis in the **GLUS** strain. Under these conditions, leaf metabolism would be more likely to reach a steady-state level for photosynthetic intermediates. The changes observed in the pattern of recovered ^{14}C metabolites in the mutant were further evidence that the lack of glutamate does reduce carbon flow through the PCO cycle (Table 12). The percent recovery of ^{14}C in serine and glycine in the mutant was only 24% of the percent recovered ^{14}C of the WT strain. In the NH_4Cl-fed WT, the amino acids were all increased in percent ^{14}C recovered when compared with the WT control.

In addition, it was clear that the operation of the PCR cycle was affected by the absence of leaf fd-dependent GOGAT, and the increased NH_4^+ content of the NH_4Cl-feeding (Table 12). In the **GLUS** plant, the percent ^{14}C recovered was lower in PGA, but increased in the SMP, RuBP and FBP fractions, when compared with WT strain. The increased

Table 12

Percent distribution of ^{14}C recovered from mature leaves of plants after 10 min of photosynthesis with $^{14}CO_2$ in 50% O_2 and 335 ppm CO_2 (remaining N_2) and 50 min after the onset of illumination. This experiment was replicated three times (average ± S.E.).

Fraction	Strain		
	WT	GLUS	NH_4Cl-fed WT
Acid-1	8.2	40.9	14.2
malate	3.5±1.2	21.0±2.1	10.1±1.0
citrate	0.4±0.1	3.4±0.1	2.0±0.4
2-oxo-glutarate	0.0±0.0	5.7±1.0	0.0±0.0
succinate	0.0±0.0	1.1±0.3	0.5±0.1
glyoxylate	0.3±0.1	3.9±0.9	0.0±0.0
Acid-2	15.4	22.9	17.0
PGA	3.2±0.9	1.0±0.4	2.2±0.9
SMP	6.2±1.0	12.3±1.3	8.1±0.7
Acid-3	6.3	11.5	3.1
RuBP	2.5±0.3	5.3±0.5	0.6±0.1
FBP	1.8±0.5	4.2±0.8	2.0±0.5
Basic	41.4	12.4	50.8
glycine	20.5±1.2	4.1±0.4	20.1±1.6
serine	14.9±1.3	4.3±0.5	16.0±1.3
glutamate	2.5±0.4	0.4±0.1	3.2±0.4
glutamine	0.3±0.1	1.9±0.1	1.1±0.2
aspartate	0.6±0.1	0.3±0.1	4.3±0.2
alanine	1.2±0.2	0.1±0.1	3.8±0.2
Neutral	15.4±1.9	7.9±1.1	8.9±1.0
Insoluble	9.1±1.0	4.9±0.9	8.2±0.9
Recovery (%)	95.8	100.6	102.2
DPM recovered (10^5)	16.4	5.45	8.96

recovery of ^{14}C in the acid-2 and acid-3 fractions was consistent with previous data obtained with the GLUS strain (Somerville and Ogren 1983). The data in Table 12 suggest that sufficient carbon was still present in the PCR cycle and accordingly corroborates the earlier RuBP study (Table 10). The increased recovery of ^{14}C in the PCR cycle intermediates of GLUS was in contrast to that of NH_4Cl-fed WT plants. Apparently, as only 3.1% of the total ^{14}C recovered was in the acid 3 fraction, versus 6.3% in the WT strain the increased NH_4^+ content of these plants (Fig. 12) may have uncoupled photophosphorylation.

The predominant products of photosynthetic CO_2 assimilation are starch and sucrose (Lilley 1983). Table 12 indicates that in the GLUS strain starch and sucrose syntheses were affected to the extent that approximately 45% less percent ^{14}C was recovered in these fractions, than in the WT strain. Only sucrose synthesis was reduced in the NH_4Cl-fed WT, by 40% of that of the WT.

Malate Metabolism in WT and GLUS Plants

Another striking change in the distribution of ^{14}C recovered in metabolites of the GLUS strain, concerned the 21% incorporation of total label into malate, versus only 3.5% in the WT and 10.1% in the NH_4Cl-fed WT (Table [illegible]). Previously, Somerville and Ogren (1983) reported no change in percent ^{14}C recovery in malate in the GLUS strain. [illegible] et al. (1984) did report an increase in labeling of malate

in the presence of high NH_4^+ in wheat, but others have found no such change in spinach leaf disks (Platt et al. 1977). Walker et al. (1984) suggested the increase in ^{14}C recovery to be a result of carboxylation of PEP. As the CO_2 exchange rate was reduced in the GLUS strain under photorespiratory conditions (Somerville and Ogren 1980a; Fig. 16), secondary carboxylations may have a large role in GLUS metabolism.

To investigate the altered malate metabolism in the GLUS strain, malate was labeled in the dark before photosynthesis was initiated. In one set of experiments, plants were fed $^{14}CO_2$ in the dark for 60 min. Under these conditions, the most highly labeled metabolites were malate, citrate, aspartate and glutamate (Table 13). The product of PEP carboxylase is OAA, which is usually metabolised to malate, aspartate and transferred into the TCA cycle (Lance and Rustin 1984). If the dark $^{14}CO_2$ products were subsequently chased in the light with a 21% O_2 and 334 ppm CO_2 (remaining N_2) gas condition, most of the ^{14}C was still recovered in the plants. Following the chase, 40% of the total ^{14}C in the GLUS strain was recovered in malate, compared to 32% in the WT strain. Thus, one pleiotropic effect on leaf metabolism caused by the absence of leaf fd-dependent GOGAT concerns an alteration of malate metabolism.

In a second set of experiments, the products derived from feeding U-^{14}C-malate directly into leaf segments were examined. As was observed in the analysis of dark $^{14}CO_2$ fixation products, essentially no difference was found in

Table 13

Percent distribution of ^{14}C recovered after 45 min of photosynthesis, after supplying $^{14}CO_2$ in the dark for 60 min in **GLUS** and WT **Arabidopsis**. The gas regime was 21% O_2 and 334 ppm CO_2 (remaining N_2). The experiment was replicated twice (average ± S.E.).

Fraction	Strain			
	WT		GLUS	
time after illumination (min):	0	45	0	45
Acid-1	55.6	44.9	55.8	58.6
malate	22.1±2.2	31.8±2.7	25.7±2.9	40.4±3.7
citrate	14.4±1.3	5.4±0.5	15.6±1.0	4.9±0.6
fumarate	1.6±0.4	0.3±0.1	0.9±0.1	1.5±0.2
succinate	7.4±0.1	4.1±0.5	5.9±0.9	3.1±0.7
2-oxo-glutarate	3.4±0.5	1.1±0.2	2.3±0.2	3.9±0.5
Acid-2	2.0	8.8	3.1	5.5
PGA	0.0±0.0	1.2±0.3	0.0±0.0	0.[illegible]±0.1
Acid-3	0.7	7.4	0.4	2.9
Basic				
glutamate	10.3±0.9	12.6±1.0	9.3±0.6	6.9±0.7
glutamine	3.7±0.3	2.4±0.1	2.3±0.2	8.4±0.6
alanine	2.8±0.4	2.5±0.4	2.4±0.2	0.9±0.2
aspartate	15.4±1.0	5.0±0.8	16.8±0.7	0.7±0.2
Neutral	0.0±0.0	7.7±0.9	0.0±0.0	4.7±0.8
Insoluble	1.6±0.3	3.6±0.8	1.4±0.2	2.0±0.2
Recovery (%)	98.4	102.6	98.5	95.6
DPM recovered (10^3)	11.4	9.94	10.5	10.1

the products from labeling by this procedure fo[illegible]0 min (Table 14). The principal products recovered [illegible] malate, aspartate and glutamate in both strains. After 30 min of illumination, many products were metabolised further, with ^{14}C recovery in sucrose equivalent between the strains. By 60 min, the percent recovery of ^{14}C was reduced in the mutant to 7.7% versus 11.7% in the WT. Also, only 22.8% of the total label was recovered in the amino acids of the **GLUS** strain, in comparison to 35.5% in the WT. The percentage of ^{14}C recovered in malate decreased with time in the WT strain from 34% to 19% but changed little (from 33% to 28.4%) in the mutant **GLUS**.

Isolation of Revertants to the GLUS Locus

In order to gain further insight as to why **GLUS** photosynthetic CO_2 assimilation is inhibited and to understand why **GLUS** plants are inviable in normal air, plants which were altered in their **GLUS** phenotype were sought. The approach used was to mutagenise a **GLUS** strain in hopes of altering either the original **GLUS** locus or another genetic locus, such that the plants would appear to be phenotypically normal. As the following revertant plants were not genetically mapped, it must be considered that they were phenotypic suppressors and not, necessarily, true revertants at the **GLUS** locus. Table 15 lists the results of an EMS-mutagenesis experiment with three **GLUS** strains, CS 37, CS 66 and CS 254. The mutagenesis was judged effective

Table 14

Comparison between **GLUS** and **WT** strains of the percent distribution of ^{14}C recovered over 90 min of photosynthesis in 21% O_2 and 334 ppm CO_2 (remaining N_2). U-14C-malate was supplied to leaf strips for 30 min, in the dark. This experiment was replicated three times and the values were averaged.

Fraction	Strain					
	WT			**GLUS**		
time after illumination (min)	0	30	90	0	30	90
Açid-1	50.3	38.9	33.0	46.6	46.7	47.4
malate	34.0	27.1	19.4	33.2	31.8	28.4
citrate	5.1	2.0	2.6	3.9	4.6	5.8
fumarate	1.3	1.0	0.4	0.7	0.9	1.8
succinate	3.2	2.0	2.1	2.8	2.4	4.6
Acid-2	2.4	9.0	7.2	1.4	6.1	9.2
PGA	0.0	0.1	0.5	0.0	0.0	0.1
Acid-3	0.0	5.0	6.8	0.0	4.1	4.3
Basic	44.0	33.6	35.6	47.6	30.8	22.9
glycine	0.1	3.1	4.2	0.0	[illegible].9	2.0
serine	0.6	3.9	4.5	0.2	2.6	3.4
aspartate	25.1	6.2	6.0	25.7	6.4	2.1
glutamine	3.5	4.2	1.3	3.0	3.9	5.3
glutamate	9.6	11.0	10.3	11.3	8.5	4.0
Neutral	0.0	5.3	11.7	0.0	6.4	7.7
Insoluble	1.0	4.1	6.6	2.1	3.2	4.6
Recovery (%)	97.9	95.9	100.9	97.3	97.9	96.8
DPM recovered (10^4)	59.5	56.5	51.4	66.0	61.9	51.9

in each case if at least one chlorophyll-streaked plant was observed per 100 plants in the M1 generation. The M1 generation was raised in a CO_2-enriched chamber and the seeds were collected "en masse". In order to screen for revertants, the M2 were sown in flats and maintained in normal air. Under the conditions of this screen, a **GLUS** mutant would germinate, but remained small (not developing past the rosette stage) and yellowish. In contrast to earlier reports with photorespiratory-deficient mutants (Somerville and Ogren 1980a; Somerville and Ogren 1982b), the **GLUS** strain used in the present study was viable for 25 to 50 days in normal air. Revertants selected from the mutagenesis of CS 37 and CS 66 contained from 71 to 134% of the fd-dependent GOGAT activity present in the WT strain (not shown). These plants were not examined any further as it was felt further study of these revertant plants would add little information as to the causes of both inviability and the low photosynthetic rate of the original mutant.

Growth Characteristics of the Revertants

As shown in Table 15, 55 revertant plants were found in the screen using the CS 254 strain (here after refered to as the original mutant, or simply the **GLUS** strain). These 55 were those plants which flowered and set seed. It is of interest to note that in screening for revertants, 331

Table 15

The results from EMS-mutagenesis on the recovery of altered phenotypes in the M1 and M2 generations of **A. thaliania**, **GLUS** strains.

Strain	Percent germination in the M1	Frequency of altered phenotypes			
		chlorophyll-streaked **M1**		phenotypic revertants **M2**	
		total counted	streaked	seeds planted	revertant
CS 37	96.0	4329	117	50,000	17
CS 66	87.9	2640	239	174,000	7
CS 254	92.5	30,360	1356	$2.4x10^6$	55

plants were actually judged to be superior in appearance to the mutant. However, only 55 plants flowered and set seed whereas 276 plants would not. Most of these latter plants were larger than the **GLUS** plants and unusually dark green. Attempts to recover seeds failed, despite various treatments with gibberellic acid, kinetin and glutamate (not shown). These plants resembled the WT strain when grown on high concentrations of NH_4Cl (Doddema et al. 1978). The nature of these "revertant" plants remains a mystery.

Table 16 lists the results of a growth study with 9 revertants of the M5 generation, from CS 254. These 9 revertants had the most easily distinguishable appearance when compared with the original mutant. The plants were studied in normal air for their growth. After germination the revertants were low in vigor, often as low as the original mutant. A noticeable difference in appearance was evident between the mutant and revertant strains after 2 to 3 weeks of growth (Table 16). The revertant plants were small in comparison to the WT strain, and the dry weight was 1.3 to 2.0 times higher than the original mutant. When grown in air, the chlorophyll content of the revertants were low (Table 16). **REV11** had the highest chlorophyll content of any of the revertants and its level was only about 50% of that of the WT strain. In CO_2-enriched air (as judged on chlorophyll content) the revertants and the **GLUS** str[illegible] as well as the WT strain.

Table 16

Growth and chlorophyll content of revertant plants from CS 254 (GLUS) raised in normal air and in CO_2-enriched growth chambers. The plants were 27-33 days old and the experiment was repeated once. The dry weight measurements were from plants grown in normal air.

Strain	Chlorophyll content (μg chl. mg fw^{-1})		Dry weight (mg $plant^{-1}$)
	normal air	CO_2-enriched air	
WT	1.510	1.39	15.20
GLUS	0.164	1.30	3.70
REV1	0.300	1.29	N.D.
REV2	0.476	1.27	5.04
REV4	0.440	1.48	5.60
REV6	0.28	1.29	N.D.
REV7	0.382	1.39	6.30
REV8	0.28	1.25	N.D.
REV11	0.672	1.32	7.50
REV36	0.32	1.24	N.D.
REV45	0.296	1.21	5.80

N.D. = not determined

GOGAT, GDH and RuBISCO Activities in the Revertants

Neither the amount of fd-dependent GOGAT activity nor NADH-dependent GOGAT activity in the reverants differed substantially from that of the original mutant (Table 17). The amount of GDH activity or RuBISCO activity were also determined and were found not to be altered.

It was decided that further experiments would be done with **REV11** in order to determine what changes in its metabolism might be responsible for its increased growth and chlorophyll content. Therefore, GDH was partially purified and its Km for NH_4^+ determined, as this enzyme does have a key role in nitrogen metabolism and could supply glutamate to the PCO cycle (Wallsgrove et al. 1983). Partially purified GDH from **REV11** had a Km for NH_4^+ of approximately 27 mM. The WT strain's GDH had a Km of 29 mM. Both Km values are close to values reported elsewhere (Pahlich and Joy 1971; Stewart and Rhodes 1977) and are suggestive of the fact that GDH has not been altered in this revertant.

Genetic Analysis of REV11

The **REV11** line was judged distinct enough in appearance, that if the plant was crossed into a WT strain, the F_2 progeny should segregate into distinct phenotypic classes. Yellowish plants did segregate in the F_2 generation and were considered to be diploid for the original CS 254

Table 17

Initial survey of revertants for various enzyme activities. Plants were 28-36 days old. The values were averaged from three replicate experiments.

Strain	Enzyme Activity				
	GOGAT		RuBISCO	GDH	
	NADH	fd		NADH	NAD
	(μmoles product mg protein^{-1} hr^{-1})				
WT	0.29	6.0	8.0	4.2	1.8
GLUS	0.25	0.38	5.8	4.3	1.7
REV2	0.24	0.20	5.7	3.9	1.6
REV4	0.25	0.64	5.9	3.9	1.6
REV7	0.25	0.10	6.1	3.4	1.9
REV11	0.29	0.25	5.6	4.3	1.6
REV45	0.25	0.13	5.7	4.1	1.7

allele (**gluS**) and REV$^+$. An intermediate phenotype was observed, and these plants were classified as homozygous for the CS 254 allele and homozygous at the revertant locus. In the F_2, 160 plants were WT in appearance (with fd-dependent GOGAT activity), 32 were yellow (with no fd-dependent GOGAT activity) and 12 were lime-green in leaf color (with no fd-dependent GOGAT activity). In a true dihybrid cross four phenotypic classes would be expected. As this was not observed, the data were indicative of the recessiveness of the revertant allele. A chi-square test of the data gave a value of 18.4, when tested for an expected 4 phenotypic classes. This value is not acceptable at the 0.05 level of significance. Alternatively, the REV$^+$ locus is epistatic to the **GLUS**$^+$ locus and only three phenotypic classes would be expected in the F_2 generation. They are: the mutant class (homozygous for **gluS**$^-$ alleles), the revertant class (homozygous for both **gluS**$^-$ alleles and **rev11** alleles) and a WT class (which contains both homozygous **rev11** and **rev**$^+$ alleles respectively). A chi-square test of this hypothesis revealed the data to yield a value of 1.34, which is acceptable at the 0.05 level of significance. To test for allele-specificity of the **REV11** strain, it was crossed to the CS 66 allele of the **GLUS** locus. In the F_1, all the plants were yellowish, again suggesting that the **REV11** locus was recessive. In the F_2, 9 light green and 96 yellowish plants were counted. The locus responsible for the **REV11** phenotype does not appear to be linked to the **GLUS**

locus. Moreover, it must be considered that this revertant strain was a phenotypic suppressor to the original mutant.

Photosynthetic CO_2 Exchange Rates of REV11

Since the **REV11** strain did not represent a true revertant, its characteristics called for further investigation in expectation that studies with this strain might shed light on the causes for inviability and inhibition of photosynthetic CO_2 exchange in the **GLUS** class of photorespiratory-deficient mutants. Figure 18 presents the CO_2 fixation curve for the WT, **GLUS** and **REV11** strains. The revertant strain was still susceptible to inhibition under a photorespiratory gas regime. Photosynthesis of the revertant strain was only marginally higher than a the **GLUS** plant. After 60 min of photosynthesis, the WT rate was 70.5 µmoles CO_2 fixed mg chl^{-1} hr^{-1}, for the **GLUS** strain the photosynthetic rate amounted to 33 whereas for the **REV11** strain the value amounted to 39. A characteristic of the **REV11** strain was the quicker rate of photosynthetic recovery from inhibition in the dark when compared to the **GLUS** strain. The events leading to the restoration of photosynthesis of the **GLUS** strain and of the other photorespiratory defective mutants of **A. thaliania** were not further examined (Somerville and Ogren 1982c). Since the difference in photosynthetic rate between the mutant and revertant appeared to be marginal, it was decided that in

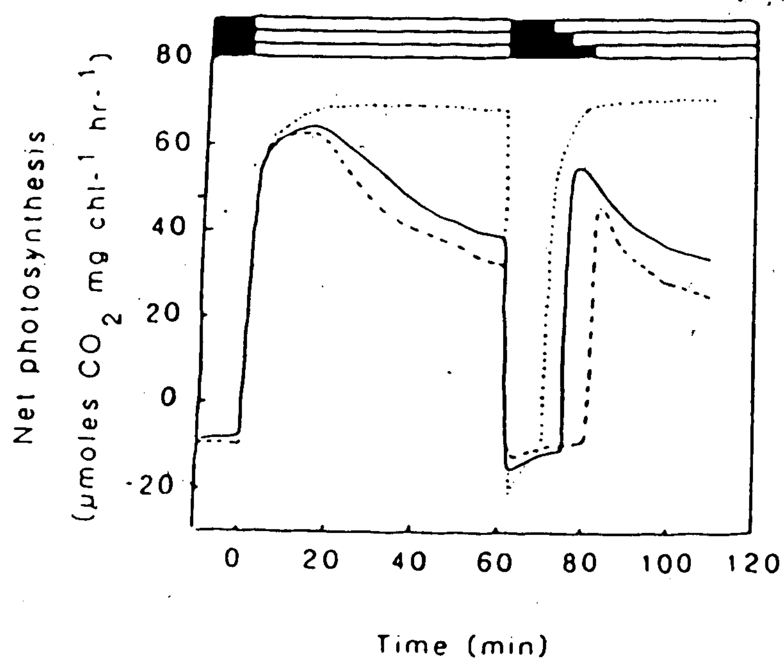


Figure 18 Rates of net photosynthesis in WT (· · ·), **GLUS** (- - -) and **REV11** (—) plants in 21% O_2 and 340 ppm CO_2 (remaining N_2). The photosynthetic data were collected for 4 to 7 plants, for each strain.

order to distinguish further the two strains other physiological characteristics should be examined.

NH_4^+ Levels in REV11

The accumulation of NH_4^+ under photorespiratory conditions (Somerville and Ogren 1980a; Fig. 17) is a characteristic of the GLUS strain. The NH_4^+ levels for the three strains measured after various times of photosynthesis in 21% O_2 and 340 ppm CO_2 (remaining N_2) are given in Figure 19. The REV11 strain accumulated about 30% more NH_4^+ than the GLUS strain did after 60 min of photosynthesis. Consequently, REV11 plants have not gained the ability to assimilate NH_4^+. The higher NH_4^+ content of the REV11 strain was probably due to the higher rate of photosynthesis under photorespiratory conditions. When the GLUS and REV11 strains were given a 40 min dark period, the NH_4^+ level declined by about 20% in each strain. Although this figure constitutes a rather crude estimate, it seems reasonable to conclude that neither strain differed in dark assimilation of NH_4^+, or the diffusion of NH_4^+ out of the leaf.

$^{14}CO_2$ Radiolabeling of Metabolites in REV11

It has been suggested that the decline in the photosynthesis in GLUS plants is caused by a block in the PCO cycle (Somerville and Ogren 1983; Creach and Stewart 1982). To determine as to whether or not the PCO cycle was blocked in some revertant strains, plants were labeled with

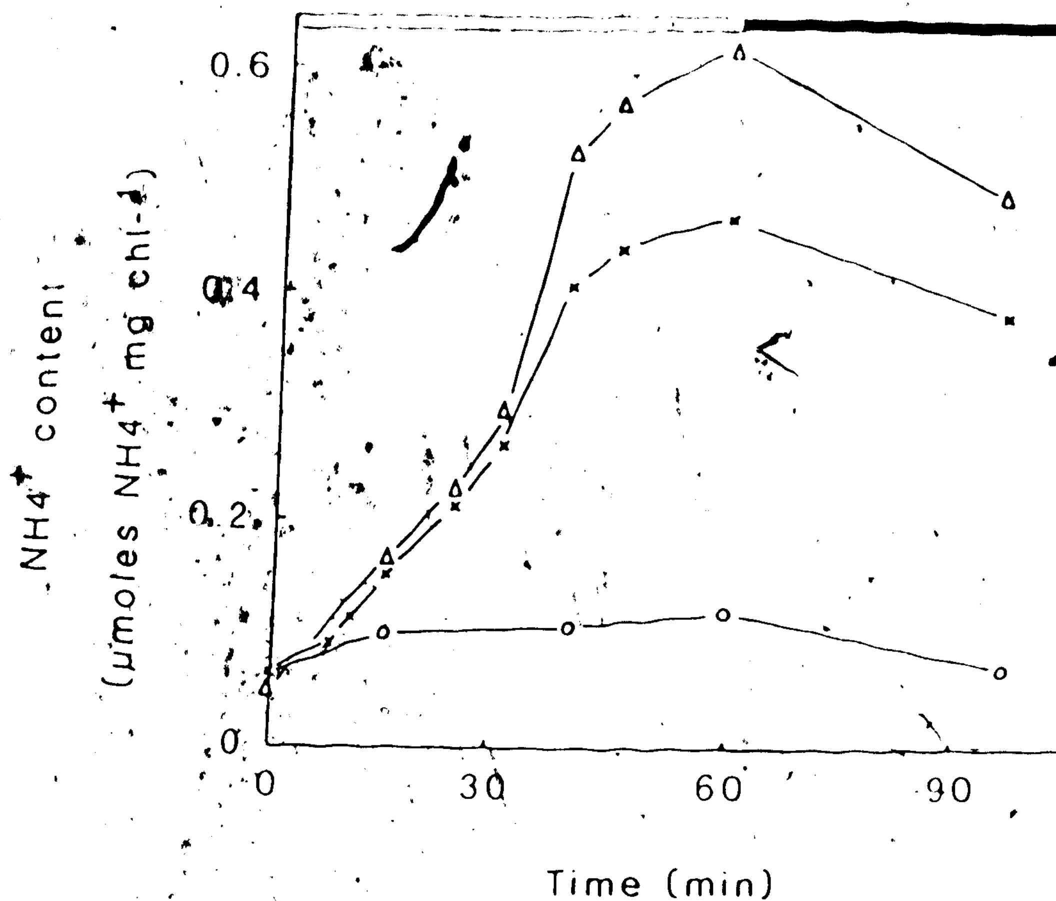


Figure 19 The NH_4^+ content of WT (o), GLUS (x) and REV11 (Δ) leaves from plants photosynthesizing in 21% O_2 and 340 ppm CO_2 (remaining N_2). The NH_4^+ data were collected from at least 9 plants per data point shown. This experiment was repeated with plants from different seeding dates. The data points were averaged.

$^{14}CO_2$ and the photosynthetic products analysed. No differences from the **GLUS** strain were found in experiments conducted in 50% O_2 and 345 ppm CO_2 (remaining N_2) (Table 18). A distinguishing feature of the **GLUS** strain under these conditions was the low recovery in percent ^{14}C in glycine and serine which are indicative of a block in the PCO cycle (Somerville and Ogren 1983). No change to this pattern was observed in the revertants. Table 19 gives more detailed information with respect to the ^{14}C recovery of metabolites of the WT, **GLUS** and **REV11** strains in 2% and in 21% O_2 respectively. In 2% O_2, no difference in metabolism between any of the strains was found. Thus, the deficiency in fd-dependent GOGAT activity was apparent only under conditions allowing photorespiration. The labeling pattern obtained in 21% O_2 was considerably different and, yet, characteristic for mutants defective in the operation of the PCO cycle (Somerville and Ogren 1982a). In this respect, the **REV11** line appeared to be identical to the original mutant, even though it attained a higher dry weight than the **GLUS** strain, when grown in normal air (Table 16).

Amino Acid Levels in REV11 Plants

The lack of leaf fd-dependent GOGAT activity in **GLUS** plants decreases the total amino acid content when photosynthesizing in 50% O_2 and 350 ppm CO_2 (Somerville and Ogren 1980a). Somerville (1984) proposed that the lack of glutamate reduces **GLUS** viability, because glutamate is a

Table 18

The percent recovery of ^{14}C from revertant plants labeled with $^{14}CO_2$ in 50% O_2 and 345 ppm CO_2. The plants were labeled from the 5-15 min interval after the onset of illumination. This experiment was replicated 3 times. The values were averaged.

Fraction	Strain					
	WT	GLUS	REV2	REV4	REV7	REV11
Acid-1	13.9	18.9	17.7	23.0	21.9	22.0
malate	3.4	4.9	4.7	3.0	3.3	5.0
Acid-2	18.5	22.0	20.7	21.9	23.8	23.4
Acid-3	8.9	14.0	15.1	14.1	14.8	16.8
Basic	34.6	17.9	18.5	18.1	19.2	18.9
alanine	1.6	1.4	0.5	1.2	0.9	0.5
glutamine	1.0	2.3	1.1	1.8	1.9	1.1
glutamate	0.2	1.2	1.0	2.6	1.9	2.3
glycine	18.0	7.2	8.4	8.1	7.8	9.0
serine	13.1	4.9	5.2	5.1	6.0	5.8
Neutral	13.5	9.4	10.7	11.2	9.9	10.1
Insoluble	11.6	8.0	9.1	11.3	7.5	8.3
Recovery (%)	101.0	90.8	92.8	99.6	97.1	99.1

Table 19

Percent distribution of ^{14}C recovered after 10 min of photosynthesis in 21% O_2 and 340 ppm CO_2 and after 10 min in 2% O_2 and 360 ppm CO_2 in the WT, GLUS and REV11 plants of Arabidopsis. Plants were labeled at the 11-22 min interval after the onset of illumination. This experiment was replicated twice. The values were averaged.

Fraction	2% O_2			21% O_2		
	WT	GLUS	REV11	WT	GLUS	REV11
Acid-1	9.9	11.3	8.1	9.7	19.6	21.1
malate	2.4	2.7	2.1	3.4	8.7	10.3
citrate	1.9	2.0	1.6	1.3	3.4	1.6
glyoxylate	0.0	0.0	0.0	0.0	1.0	1.6
2-oxo-glutarate	[illegible].0	0.2	0.3	0.0	2.0	2.8
Acid-2	17.9	17.8	18.4	13.6	21.8	19.7
PGA	6.5	7.0	7.2	4.5	7.9	6.3
SMP	5.1	6.3	6.7	5.0	10.2	12.2
Acid-3	5.5	6.5	6.1	9.4	19.0	18.0
RuBP	2.7	2.1	2.6	2.1	8.6	8.1
Basic	19.9	19.5	20.3	34.8	19.4	18.4
glycine	1.2	2.0	1.9	12.9	9.3	6.1
serine	6.1	5.7	6.0	11.1	4.1	3.5
glutamate	0.0	0.0	0.1	3.4	0.9	1.1
glutamine	0.0	0.0	0.1	0.1	2.0	2.2
alanine	2.6	1.9	1.4	2.0	0.5	0.1
aspartate	3.0	2.7	2.6	1.4	0.6	0.3
Neutral	27.6	24.7	23.4	15.2	12.7	11.4
Insoluble	17.2	15.8	16.4	9.9	7.9	7.0
Recovery(%)	98.0	95.6	93.2	94.2	100.0	95.2

precursor of chlorophyll (Beale 1978). In order to discover as to whether or not the **REV11** mutation affected leaf amino acid levels, plants were allowed to photosynthesize in 50% O_2 and 349 ppm CO_2 after which their amino acid content was determined. Typically, for each strain, 6 to 10 plants were used per time point. Due to a shortage of time it was necessary to use plants from 2 growth chambers and the data obtiained from these two sets of plants are given in Tables 20 and 21. At the start of the experiment (when the plants have pre-equilibrated in the dark for 30 min), the leaf amino acid content was similar between the WT, **GLUS** and **REV11** strains (if comparison is kept within a set of plants originating from one and the same growth chamber). These results were in contrast with results obtained with the **DCT** mutant of **A. thaliania**, in which glutamate and glutamine metabolism in the dark are quite different between the mutant and WT strains (Somerville and Ogren 1983). Although the absolute amounts were not comparable, the data in both, Table 20 and Table 21, suggested a decline in glutamate and an increase in glutamine in the **REV11** and **GLUS** strains when compared with WT approximately 10 min after the onset of illumination in 50% O_2 and 349 ppm CO_2. This trend continued for later time points. In addition, the **REV11** strain appeared to have a higher glutamate content then the **GLUS** strain, suggesting an increased synthesis of glutamate in the leaf of **REV11** plants during photorespiration. This observation was, at odds with the enzymatic data, where no

Table 20

The effects of a photorespiratory gas regime of 50% O_2 and 349 ppm CO_2 on the levels of amino acids in leaves of WT, **GLUS** and **REV11** **Arabidopsis**. Plants were raised in growth chamber number 1. The plants were 25 to 28 days old.

Amino Acid (µmoles/ mg chl)		Time (min)			
			10	20	40
ALA	**WT**	0.7	0.56	1.15	0.90
	GLUS	0.5	0.61	0.73	0.45
	REV11	1.0	0.92	0.81	0.12
ASP	**WT**	1.3	1.36	1.35	0.97
	GLUS	0.9	0.88	0.70	0.47
	REV11	1.1	1.09	0.88	0.42
AXN	**WT**	0.3	0.34	0.36	0.33
	GLUS	0.3	0.28	0.30	0.23
	REV11	0.7	0.63	0.45	0.36
GLU	**WT**	2.5	3.48	5.03	5.77
	GLUS	2.6	3.02	1.31	0.97
	REV11	2.8	3.28	1.74	0.50
GLN	**WT**	1.2	1.21	1.76	2.05
	GLUS	1.3	1.81	2.32	2.58
	REV11	1.3	1.99	2.02	2.45
GLY	**WT**	0.8	1.80	2.45	3.28
	GLUS	0.9	1.07	1.42	0.63
	REV11	0.5	1.26	0.95	0.92
SER	**WT**	0.5	1.43	2.45	3.28
	GLUS	0.9	1.27	1.51	0.51
	REV11	0.6	0.77	0.92	0.38

Table 21

The effects of a photorespiratory gas regime of 50% O_2 and 349 ppm CO_2 on the levels of amino acids in leaves of WT, GLUS and REV11 Arabidopsis grown in growth chamber two. The plants were 25 to 29 days old.

Amino acid (μmole $mg\ chl^{-1}$)		TIME 0	10	[illegible]	40
ALA	WT	1.3	0.83	0.79	0.69
	GLUS	0.7	0.66	0.59	0.37
	REV11	0.9	0.81	0.76	0.27
ASP	WT	0.8	0.73	0.81	0.69
	GLUS	1.1	0.99	0.29	[illegible]
	REV11	0.8	0.63	0.38	0.23
ASN	WT	0.2	0.27	0.32	0.21
	GLUS	0.4	0.39	0.3[illegible]	0.18
	REV11	0.4	0.45	0.26	0.14
GLU	WT	2.9	2.84	3.72	4.29
	GLUS	2.2	2.28	1.67	1.04
	REV11	2.2	2.42	1.91	1.38
GLN	WT	0.8	0.93	1.52	2.70
	GLUS	1.7	2.00	2.89	2.05
	REV11	1.3	2.42	2.68	2.71
GLY	WT	0.7	1.80	2.11	2.89
	GLUS	0.6	1.34	1.12	0.59
	REV11	0.6	1.23	0.95	1.13
SER	WT	1.1	1.67	1.94	1.95
	GLUS	1.3	1.46	1.20	0.89
	REV11	0.6	1.14	0.91	0.56

difference was found in the NADH- or fd-dependent GOGAT activity (Table 17).

The glutamate levels measured at a time when photosynthesis starts to decline were higher than reported previously with the **GLUS** strain (Somerville and Ogren 1980a). However, the corresponding amino acid levels measured were similar to those reported with the **DCT** mutant when treated in the light (Somerville and Ogren 1983). The plants used in this study generally required an additional 7 to 10 days of growth in order to reach the rosette stage when compared with the plants reported previously by Somerville and Ogren (1980). Therefore, the differences in amino acid levels may reflect the different growth conditions of the two sets of experiments which in turn could have resulted in a larger vacuolar pool size. It is known that a large reservoir of amino acids does exist in the vacuolar compartment of the cell (Matile 1980). Nonetheless, there was clearly a deficiency of glutamate in the leaves of **GLUS** and **REV11** plants compared to the WT after photosynthesizing in photorespiratory conditions. This was supported by the observation that there was a reduced ^{14}C recovery in glycine and serine under photorespiratory gas conditions (Tables 18 and 19).

Effects of NH_4^+ on REV11 Chloroplast Photosynthesis

Although no conclusive role for NH_4^+ in causing photosynthetic inhibition in the **GLUS** or **DCT** strains has

been established in other studies (Somerville and Ogren 1983; Somerville 1984), the present author decided to examine the effects of NH_4^+ on chloroplast photosynthesis. Chloroplasts were isolated from each strain and tested for photosynthetic rate in the presence of NH_4^+. The data indicate that NH_4^+ inhibited photosynthesis, and equally in all the strains (Table 22). In 10 mM $NaHCO_3$, approximately 2.00 mM NH_4Cl inhibited photosynthesis by 50%. However, in 340 ppm CO_2 (about 20 µM CO_2) 0.3 mM NH_4Cl inhibited photosynthesis by 50% (Table 23). None of the strains showed a difference in the susceptibility of chloroplasts to NH_4Cl inhibition and it appears that at least at the chloroplast level REV11 plants were not more resistant to the presence of NH_4^+.

RuBISCO Activation Levels in REV11 Plants

The inactivation of RuBISCO was indicative of the inhibition of photosynthesis of plants blocked in the PCO cycle (Creach and Stewart 1982; Chastian and Ogren 1985). This possibility was examined in this thesis and no direct role was found for the inactivation of RuBISCO and the inhibition of photosyntetic CO_2 exchange (compare Fig. 14 to Fig. 15). Nevertheless, there is a possibility that the RuBISCO activity was higher in the revertant than in the GLUS strain. It was found, however, that RuBISCO declined with time in the revertant, in a fashion similar to that of

Table 22

The effects of NH_4^+ on CO_2-dependent O_2 evolution of intact chloroplasts isolated from WT, **GLUS** and **REV11** plants in 21% O_2 and 8.5 mM $NaHCO_3$. The range of intactness for the chloroplasts was 82 to 96%, as measured by the ferricyanide test. The experiment was replicated 3 times and the values were averaged (± S.E.).

NH_4Cl (mM)	WT	GLUS	rev-11
	(μmoles O_2 mg chl^{-1} hr^{-1})		
0.00	96.[illegible]±[illegible].3	92.6±5.6	90.3±5.5
0.10	98.8±5.8	85.9±6.3	85.9±5.4
0.25	95.8±5.5	81.6±5.6	85.8±6.0
0.50	91.7±5.8	75.1±5.5	79.4±5.3
1.00	67.0±5.5	56.4±4.9	60.8±5.0
2.00	49.1±4.7	47.2±4.8	49.7±4.6
5.00	0.0	0.0	0.0

Table 23

The effects of NH_4^+ on CO_2-dependent O_2 evolution of intact chloroplasts isolated from WT, GLUS and REV11 plants. The gas regime used was 21% O_2 and 340 ppm CO_2. The same batch of chloroplasts reported on was used for the experiment reported on in Table 22.

NH_4Cl (mM)	WT	GLUS	REV11
	(μmoles O_2 mg chl^{-1} hr^{-1})		
0.00	34.5±3.8	32.1±4.0	31.7±4.1
0.05	31.4±3.9	29.7±4.1	30.0±3.8
0.10	28.6±3.6	27.3±4.0	26.7±3.7
0.20	20.7±3.0	22.4±3.2	21.0±3.3
0.50	10.1±1.2	9.8±1.0	10.1±1.5
1.00	4.7±0.8	4.1±0.5	5.0±0.6

the GLUS strain (Table [illegible]).

Aspects of REV11 Physiology

The GLUS strain was isolated from a mutant selection procedure, which was conceptually based on the notion that if a block in the PCO cycle were to occur certain toxic intermediates would accumulate (Somerville and Ogren 1982a). The marginally increased dry weight of the [illegible] strain in air, could be the result of an increased tolerance to [illegible] proposed toxic intermediates. A striking feature of [illegible] exposed to a stress (e.g. low cellular water potential or too high a light exposure), consists of a translation of this stress into a damaged photochemical apparatus (Bjorkman 1980). This damage is manifested by an altered response of photosynthesis to light intensity.

As shown in Figure 20, photosynthesis was markedly influenced by light intensity in **A. thaliania**. Typically, at low intensity photosynthetic CO_2 assimilation was linearly related to light intensity. Once the limit for CO_2 assimilation is reached, photosynthesis plateaus (Bjorkman 1980). In 2% O_2 and 360 ppm CO_2 (remaining N_2), following 2 days of exposure to normal air, the **GLUS** strain's photosynthetic CO_2 exchange rate reached a maximum rate at a lower light intensity than the WT. On the other hand, the **REV11** strain appeared to be very similar to the WT in its photosynthetic response to light intensity. When the light

Table 24

The activity of RuBISCO in WT, **GLUS** and **REV11** plants, photosynthesizing in 50% O_2 and 335 ppm CO_2 (remaining N_2). The plants were grown in CO_2-enriched air. This experiment was carried out once. The plants were 28 days old.

Time (min)	WT	GLUS	REV11
	RuBISCO Activity (μmoles CO_2 mg chl^{-1} hr^{-1})		
0	65.2	62.1	68.7
2.5	91.2	71.2	73.5
5.0	112.8	84.8	94.4
10.0	128.3	100.7	111.6
15.0	139.6	98.1	113.2
20.0	124.9	93.5	104.8
40.0	130.3	77.1	92.0
70.0	123.2	72.2	84.3

reactions were limiting to photosynthesis, there was no difference in photosynthesis between any of the strains, as the initial slopes appeared to be similar. The curves (Fig. 20) were developed by varying light intensity with layers of cheese-cloth. It is possible that under these experimental conditions a significant light scattering occurs. Therefore, in order to determine the light response of photosynthesis more accurately a future study should incorporate the use of higher-quality light filters.

The effects of the photorespiratory stress on the metabolism of GLUS and REV11 plants were further investigated. Plants were raised in CO_2-enriched air and then exposed to normal air for up to 6 days. From the results observed, it was obvious that the two mutant strains were in a stressed condition. Chlorophyll and protein content decreased over the 6 days, whereas little change in chlorophyll and protein levels were measured in the WT strain (Table 25). By the second day, photosynthetic CO_2 exchange rate in 2% O_2 decreased by 14% in the GLUS strain, but was unchanged from day 0 in the REV11 plants (Table 25). By the fourth day, photosynthetic CO_2 exchange was 61% in the GLUS strain and 90% of the day 0 rate in the REV11 strain. Hence, it appeared that the photosynthetic apparatus was inhibited in the original mutant strain. In contrast, protein and chlorophyll losses were more gradual in the GLUS strain, since on day 2 little difference in protein or chlorophyll was obvious when compared with their levels on

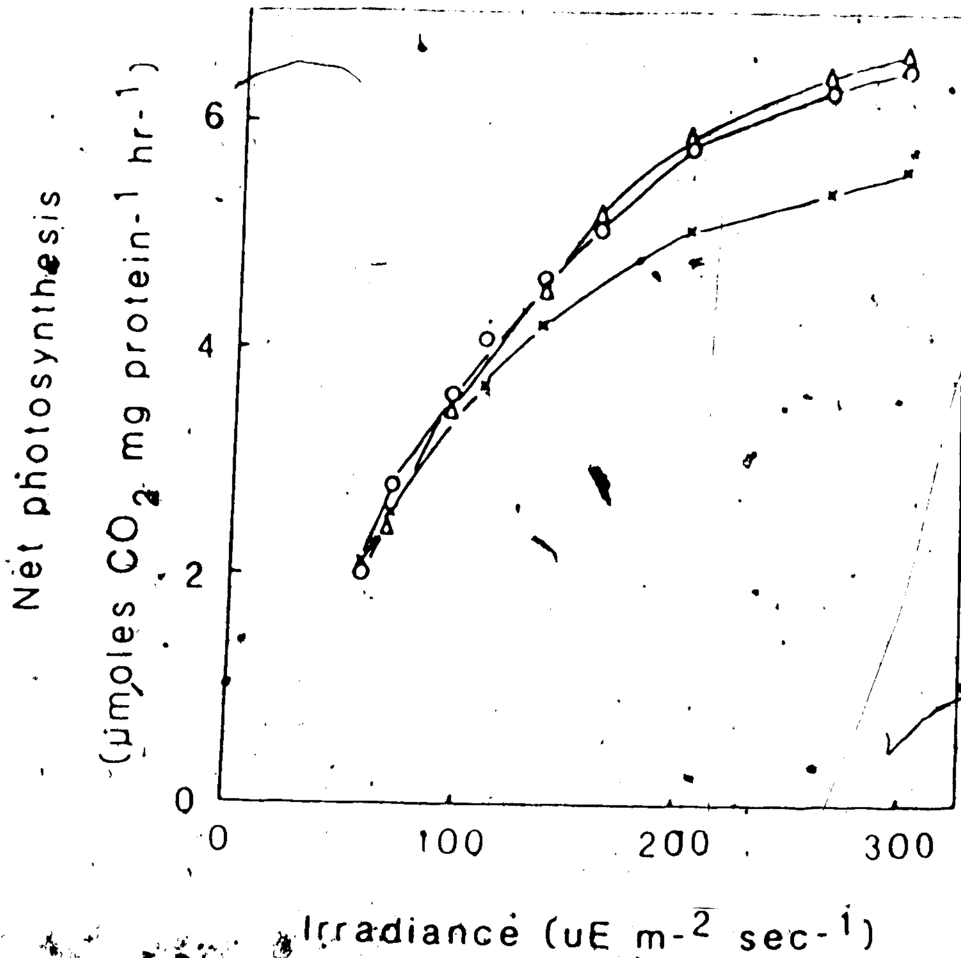


Figure 20 Rate of net photosynthesis in WT (Δ), GLUS (x) and REV11 (o) plants, plotted against light intensity measured at the leaf surface. This experiment was repeated with plants from different seeding dates. The data in the figure were averaged.

day 0. In addition on day 2, there was little difference between the **GLUS** and **REV11** strains in protein or chlorophyll content. Initially, the dark CO_2 exchange rate, or the respiration rate, in the **GLUS** and **REV11** strains when compared with the WT rate increased over the course of the experiment, after which they declined,

Isolated thylakoids from **GLUS** and **REV11** plants showed similar trends as found for the whole plant photosynthetic CO_2 exchange. For instance for the **GLUS** strain (Table 26), electron flow from water to $K_3Fe(CN)_6$ was reduced by 79% and 54% by day 2 and 4 respectively. Hence, on the basis that protein and chlorophyll levels were not as reduced (Table 25), the decline in photosynthetic CO_2 exchange was principally due to the capture of light energy. The electron flow from water to methyl viologen in the **GLUS** strain was only reduced by 68% by day 4. This result suggests that the decline in the light energy capture may not be random but selective for a given photosynthetic process. **REV11** also lost photosynthetic transport capability, but not as quickly as observed in the **GLUS** strain. Over the course of this experiment, there was little change in WT electron transport characteristics.

In order to further document the changes in leaf physiology, various enzymatic activities were followed over the 6 day photorespiratory stress period. Stress did cause changes in activity for a number of enzymes (Table 27). RuBISCO was only slightly affected by day 2 in the **GLUS**

Table 25

The physiological characteristics of WT, GLUS, and REV11 plants grown in CO_2-enriched air and subsequently to normal air for up to 6 days. Four to ten plants were studied and the experiment was replicated once. The values were averaged (± S.E.).

	Time (days)			
	0	2	4	6
Chl[1]				
WT	1.38±0.11	1.45±0.10	1.41±0.12	1.36±0.09
GLUS	1.40±0.13	1.26±0.13	0.94±0.11	0.64±0.07
REV11	1.44±0.10	1.34±0.10	1.22±0.10	1.03±0.09
Protein[2]				
WT	24.5±1.2	24.5±1.3	24.6±1.1	23.2±1.5
GLUS	24.2±1.4	21.9±1.5	19.0±1.3	15.0±1.4
REV11	23.7±1.0	23.6±1.4	21.4±1.2	20.4±1.9
CO_2-fixed:[3]				
in 2% O_2:				
WT	6.13±0.20	6.23±0.23	6.16±0.20	5.84±0.27
GLUS	6.15±0.24	5.33±0.21	3.78±0.22	2.27±0.45
REV11	6.05±0.27	6.14±0.23	5.46±0.24	4.71±0.35
in 21% O_2:				
WT	3.71±0.21	3.60±0.23	3.56±0.25	3.42±0.20
GLUS	1.85±0.17	1.10±0.11	0.86±0.09	0.45±0.34
REV11	2.14±0.15	1.43±0.13	1.31±0.16	1.04±0.31
Respiration[3]				
WT	0.67±0.06	0.71±0.05	0.65±0.06	0.84±0.07
GLUS	0.63±0.05	0.80±0.06	0.75±0.07	0.51±0.05
REV11	0.58±0.07	0.73±0.07	0.66±0.05	0.64±0.05

[1] μg chl mg fw^{-1}
[2] mg protein mg fw^{-1}
[3] μmoles CO_2 mg $protein^{-1}$ hr^{-1}

Table 26

Light-saturated electron transport rates of chloroplast thylakoids isolated from WT, GLUS REV11 strains of A. thaliania before and after a photorespiratory stress of up to 6 days. The rates of electron transport are expressed as umoles of O_2 consumed or evolved $mg\ chl^{-1}\ hr^{-1}$. The plants used were 24 to 31 days old. The values were averaged from two experiments (± S.E.).

Electron Transport	Days	Strain		
		WT	GLUS	REV11
H_2O to $K_3fe(CN)_6$				
	0	221±14	215±12	225±28
	2	227±16	171±20	201±17
	4	238±17	117±17	178±14
	6	208±15	74±14	139±16
H_2O to Methyl Viologen				
	0	189±17	193±20	196±16
	2	191±16	177±15	182±12
	4	190±14	133±12	170±14
	6	175±12	75±14	131±[illegible]

strain, and changed little in the REV11 or WT plants. However, by days 4 and 6, RuBISCO changes were more obvious in GLUS plants, being 80% and 64% of the day 0 values. Both NADH-dependent GOGAT and PEP carboxylase decreased in activity. This phenomenon was also evident in the GLUS strain. However, NADH-dependent GDH increased in activity during day 2 and day 4, along with NAD-dependent malic enzyme (ME). Because malate was a major product of photosynthesis in GLUS plants (Table 12), the author assayed NAD-ME enzyme. It was thought that an unusual pattern of activity might appear during stress. As these enzymes were found to be little changed in the WT strain, it therefore appeared that their activity was responding to the altered metabolism of the GLUS stressed plant. The activity for a number of the REV11 enzymes showed changes similar to that of the mutant, but less gradual. The slower decline in RuBISCO activity in the REV11 strain, when compared to the GLUS strain, would allow a higher rate of photosynthetic CO_2 exchange to be maintained in normal air than in the GLUS strain.

Intact chloroplasts were isolated from all three strains and photosynthetic rates determined by the ability of either OAA or CO_2 to use photosynthetic energy. Only day 0 and day 2 were studied (attempts to isolate chloroplasts from the mutant strains on days 4 and 6 failed). On day 0, all three strains had a similar CO_2- and OAA-dependent rate (Table 28). When photosynthetic ability was examined two

Table 27

The activity of some enzymes isolated from leaves of WT, GLUS and REV11 plants grown in CO_2-enriched air and subsequently to normal air for up to 6 days. Four to ten plants were studied and the values were averaged (± S.E.) of two experiments. The plants were 25 to 31 days old.

Enzyme Activity (µmoles product mg protein^{-1} hr^{-1})	Time (days) 0	2	4	6
RuBISCO				
WT	6.72±0.24	6.65±0.30	7.01±0.26	7.08±0.22
GLUS	6.43±0.30	6.10±0.31	5.20±0.28	4.14±0.21
REV11	6.34±0.24	6.23±0.23	5.64±0.24	5.34±0.25
PEPCase				
WT	1.36±0.09	1.44±0.07	1.31±0.08	1.35±0.07
GLUS	1.41±0.06	1.06±0.05	0.91±0.07	0.85±0.06
REV11	1.39±0.05	1.12±0.07	0.93±0.05	0.93±0.05
NADH-GDH				
WT	6.91±0.34	7.47±0.22	7.06±0.23	6.62±0.31
GLUS	7.37±0.28	9.44±0.34	7.02±0.33	5.05±0.22
REV11	7.04±0.34	9.85±0.31	8.26±0.28	5.76±0.29
NADH-GOGAT				
WT	0.41±0.05	0.30±0.04	0.38±0.04	0.34±0.05
GLUS	0.44±0.05	0.38±0.05	0.23±0.05	0.16±0.04
REV11	0.38±0.04	0.34±0.03	0.32±0.05	0.30±0.05
NAD-ME				
WT	4.26±0.30	3.93±0.26	4.00±0.30	4.17±0.26
GLUS	3.91±0.44	4.73±0.45	4.36±0.37	3.27±0.39
REV11	4.25±0.43	5.02±0.49	4.63±0.35	3.94±0.46
Catalase				
WT	20.1±1.2	18.3±1.3	23.8±1.2	19.0±1.5
GLUS	22.6±1.3	25.3±1.9	20.2±1.4	13.5±1.1
REV11	22.9±1.3	30.4±2.2	25.4±1.6	18.4±1.8

days later, both OAA- and CO_2-dependent rates in the GLUS strain had decreased whereas in the REV11 strain only the CO_2-dependent rate had decreased. The WT strain appeared unaffected by the 2 day treatment.

That REV11 was more resistant to the processes of senescence, brought on by the stress, is one possible explanation for these observations. In order to test for this possibility, plants from the three strains were exposed to normal air and placed in the dark for 6 days. Six days of darkness affected all three strains, as chlorophyll decreased by approximatley 20% in each case. These decreases affected similarly the photosynthetic capacity (Table 29). For all three strains, plants quickly lost viability when left longer than 6 days in the dark. As a further documentation of the metabolism, $^{14}CO_2$ fixation experiments were conducted after stressing plants of all three strains for 4 days in darkness. The incubation of plants for 4 days in darkness alters certain regulatory aspects of metabolism. For instance, the percentage of label recovered in starch(insoluble fraction) was roughly 50% of what was previously found (Table 19). However, no unique component was detected in the metabolism of REV11 (Table 30).

It was observed during the course of CO_2 gas exchange measurements, that CO_2 fixation in 21% O_2 and 340 ppm CO_2 decreased at a faster rate for GLUS plants when kept in the CO_2-enriched growth chamber and in the light, than for

Table 28

The CO_2 fixation and oxaloacetate reduction rates of intact chloroplasts isolated from **WT**, **GLUS** and **REV11** plants grown in CO_2-enriched air and subsequently to normal air for 48 hours. Six to ten plants were used per experiment and the plants were 25 to 30 days old. The experiment was replicated using plants from different seeding dates. The values were averaged (± S.E.).

Time (hr)	Additions	Rate of O_2 evolution WT	GLUS	REV11
		(μmoles O_2 mg chl^{-1} hr^{-1})		
0	8.5 mM $NaHCO_3$	84±7	87±4	84±4
	2.5 mM OAA	41±5	47±3	46±6
48	8.5 mM $NaHCO_3$	87±8	72±6	79±8
	2.5 mM OAA	47±4	35±5	47±6

Table 29

The effects of 6 days continuous darkness on the physiology of WT, **GLUS** and **REV11 Arabidopsis**. Plants were raised in continuous light and CO_2-enriched air for 26-28 days. The CO_2 fixation rate was based on the incorporation of $^{14}CO_2$ in the 11-22 minute interval after the onset of illumination. This experiment was carried ou[illegible]

Time (days)		0	2	4	[illegible]
dry wt[1]	WT	13	13	13	12
	GLUS	12	13	13	12
	REV11	13	13	13	12
chlorophyll[2]	WT	1.2	1.3	1.2	0.9
	GLUS	1.3	1.3	1.2	0.9
	REV11	1.2	1.2	1.1	0.9
protein[3]	WT	24	24	22	19
	GLUS	24	23	22	18
	REV11	23	24	20	19
RuBISCO[4]	WT	6.4	6.3	6.0	5.3
	GLUS	6.1	6.1	5.7	5.4
	REV11	6.3	6.3	5.9	5.2
PEPcase[5]	WT	1.6	1.5	1.2	1.2
	GLUS	1.3	1.4	1.3	1.0
	REV11	1.6	1.4	1.3	1.0
CO_2 fixed[6]:					
21% O_2	WT	3.3	3.2	2.9	2.7
	GLUS	3.0	2.9	2.7	2.4
	REV11	3.0	2.9	2.6	2.3

[1] dry wt = mg plant^{-1}
[2] chl = µg chlorophyll mg fw^{-1}
[3] protein = µg mg fw^{-1}
[4] RuBISCO = µmoles CO_2 mg protein^{-1} hr^{-1}
[5] PEPcase = µmoles CO_2 mg protein^{-1} hr^{-1}
[6] CO_2 fixed = µmoles CO_2 mg protein^{-1} hr^{-1}.

plants kept in total darkness. A plant left in a high CO_2 chamber reached a maximum rate of 36 µmoles CO_2 mg chl^{-1} hr^{-1}, whereas a plant kept in the dark reached a rate of 29 µmoles CO_2 mg chl^{-1} hr^{-1} (Fig. 21). It was probable that since photosynthate would be limiting, the increased rate of decline was caused by a lower starch content in the darkened leaf. In fact, starch levels were reduced in the darkened leaves (Table 31). The lowered photosynthetic rate in 21% O_2 and 340 ppm CO_2 cannot be related to a darkness induced lower photosynthetic capability. Photosynthesis in all three strains was similar in 2% O_2 and 347 ppm CO_2. The starch levels are approximately similar in all three strains which suggests that with regard to this aspect of starch metabolism, **REV11** was not altered. The above experiment also suggests, that starch may be an active carbon reserve in maintaining the levels of PCR cycle intermediates.

Isozyme Survey Using Starch Gel Electrophoresis

To identify other possible differences in the metabolism between the three strains, starch gel electrophoresis was used. 23 enzymes were surveyed by this procedure (Table 32). The plants were all raised in a CO_2-enriched growth chamber. The enzymes were colorimetrically stained for activity after electrophoresis. In all three strains, the enzymes assayed migrated to equivalent positions and had equivalent activities (not shown).

Table 30

Percent distribution of the products of $^{14}CO_2$ assimilation from WT, GLUS and REV11 plants stressed for 4 days in darkness. Plants were labeled at the 11-22 minute interval, after the start of illumination. The gas regime was 21% O_2 and 340 ppm CO_2 (remaining N_2). This experiment was carried out once.

Fraction	WT	GLUS	REV11
Acid-1	12.7	20.5	21.1
citrate	1.0	3.4	3.8
malate	5.2	5.5	6.5
2-oxoglutarate	0.5	3.5	4.0
glycerate	0.4	2.1	1.5
glyoxylate	0.2	2.3	3.0
Acid-2	14.9	20.7	24.0
PGA	3.7	1.9	2.9
PEP	1.9	2.2	3.4
Acid-3	9.1	14.2	15.9
RuBP	2.8	5.4	4.9
Basic	38	23.5	22.6
glycine	15.0	6.4	6.7
serine	8.1	11.2	10.2
glutamate	7.6	0.5	1.3
glutamine	0.2	3.1	1.0
alanine	1.0	0.1	0.2
aspartate	2.4	0.2	0.8
Neutral	15.4	10.9	11.8
Insoluble	5.6	3.5	4.6
Recovery (%)	95.9	93.5	99.7

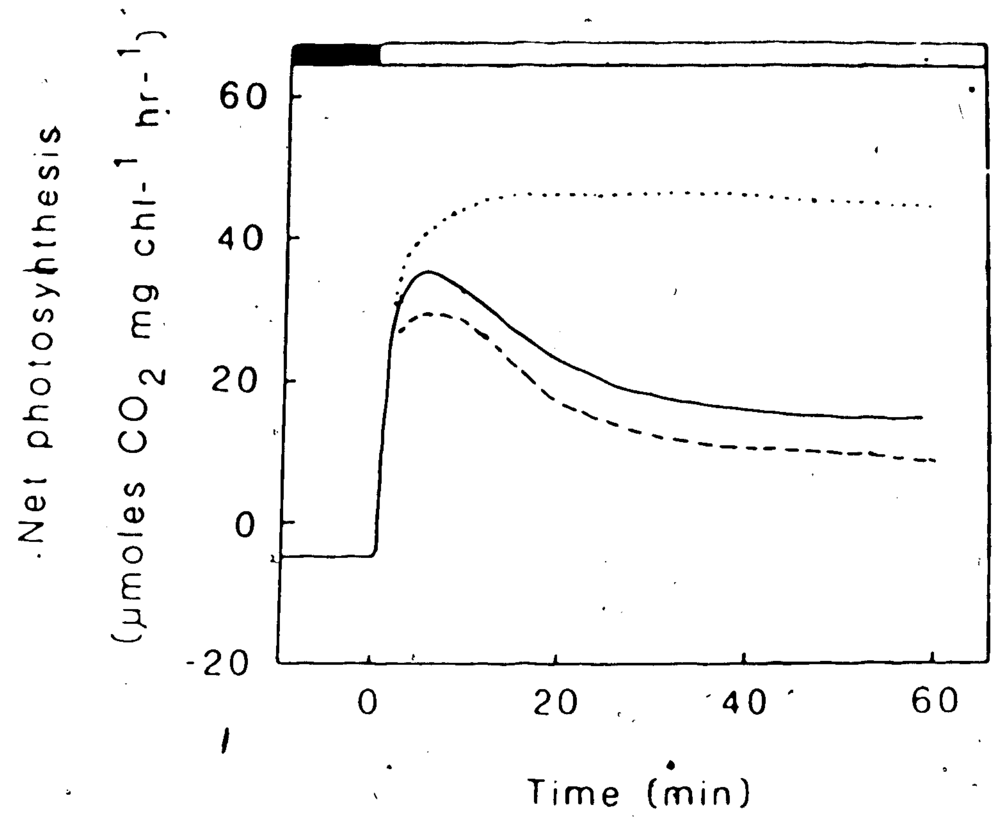


Figure 21 Rates of net photosynthesis in control grown **GLUS** (——) and dark incubated for 36 hr **GLUS** (- - -) plants. The WT rate is indicated from plants placed in the dark (· · ·). The gas regime was 50% O_2 and 360 ppm CO_2 (remaining N_2).

Table 31

The starch content and photosynthetic rate for WT, GLUS and REV11 plants raised in CO_2-enriched air and then placed in the dark for 36 hours. The gas regime for measurements of CO_2 fixation was 2% O_2 and 347 ppm CO_2 (remaining N_2). This experiment was replicated twice.

Strain	Starch content (mg glucose mg chl^{-1})		CO_2 fixation rate (µmoles CO_2 mg chl-1 hr-1)	
time (hours)	0	36	0	36
WT	43.8	17.5	101.3	109.0
GLUS	45.5	16.4	93.6	102.4
REV11	41.4	14.9	96.2	103.1

Table 32

Enzymes assayed following starch gel electrophoresis of extracts prepared from WT, GLU5 and REV11 leaves. The number of electrophoretic bands observed is given opposite the enzyme name. This experiment was replicated twice.

Enzyme	number of subunits
acid phosphatase	1
aconitase	1
adenylate kinase	2
alcohol dehydrogenase	2
aldolase	2
aspartate aminotransferase	2
catalase	2
colorimetric esterase	4
flourescent esterase	2
glucose-6-phosphate dehydrogenase	1
glutamate dehydrogenase	5
isocitrate dehydrogenase	1
leucine amino peptidase	1
malate dehydrogenase	3
malic enzyme	1
NADH diaphorose	2
peptidase	2
phosphoglucomutase	1
6-phosphogluconate dehydrogenase	2
phosphoglucose isomerase	2
shikimate dehydrogenase	1
superoxide dismutase	1
tetrazolium oxidase	2

Discussion

The GOGAT Activities of B. napus

Four reductant dependent GOGAT activities in the leaf and root tissues of B. **napus** were determined (Table 1). In the etiolated leaf, the NADH-dependent GOGAT activity predominates, whereas upon illumination of the leaf, fd- and methyl viologen-dependent activities dominated. Plants vary considerably in the amounts and types of activities found in leaf and root tissue. In rice, both NADH and NADPH activities were found only in etiolated leaf, but not in green leaf tissue (Suzuki et al. 1982). As reported herein, and by other authors for barley and pea, all 4 activities were found in both etiolated and illuminated leaves (Wallsgrove et al. 1984). Whether rice is unique in gene regulation awaits molecular studies. The fd-dependent GOGAT protein of rice however is unique when compared with other isolated fd-GOGAT'S, since it consists of two subunits (Suzuki and Gadal 1982).

It is not known which GOGAT protein is responsible for the methyl viologen activity. Matoh et al. (1980) purified leaf NADH-dependent GOGAT to homogeneity and reported both reductants, NADH and methyl viologen, gave activity. Cullimore and Sims (1981) whilst working with C. **reinhardii** partially purified NADH- and fd-dependent GOGAT activities, reported methyl viologen was only active with the NADH form

of GOGAT. Other work with the GOGAT activities of C. **reinhardii** contradict the above results. Galvan et al. (1984) isolated fd-dependent GOGAT to homogeneity, and found methyl viologen and ferredoxin to yield near equal activities. Subsequently, the same laboratory partially purified NADH-GOGAT, and also reported methyl viologen-dependent activity (Marquez et al. 1984).

In the present work, purified NADH-dependent GOGAT did not have activity with methyl viologen. Only purified fd-dependent GOGAT was shwon to have methyl viologen-dependent activity. Also, antibody raised against spinach fd-dependent GOGAT, will not inhibit NADH-activity, but will inhibit methyl viologen activity (Fig. 13). Similar results have been found in rice (Suzuki et al. 1982). It is concluded that the methyl viologen-dependent activity was caused by the same protein responsible for fd-dependent GOGAT activity.

In view of GOGAT's role in nitrogen metabolism, it is important to understand how the different forms of the enzyme function and are regulated. As reported above, the kinetics for NADH- and fd-dependent GOGAT are similar. Neither enzyme demonstrated any regulatory characteristics. This is in contrast with the characteristics of chloroplast enzyme glutamine synthetase. Both Mg^{2+} and ATP influence glutamine synthetase activity in such a way suggestive of the fact that the chloroplast physiology can affect glutamine synthetase activity (Oneal and Joy 1974; Hirel and

Gadal 1980). There seems to be no difference in pH optimum between the leaf NADH and fd-GOGAT activities, and hence it is impossible to state as to whether or not one isozyme is favored over the other during the light induced rise in stromal pH, from 7.2 to 8.0 (Portis and Heldt 1976). The only distinct difference between the two isolated GOGAT's is the requirement for either ferredoxin or for NADH. Ferredoxin is photosynthetically reduced, and therefore it is possible that the fd-dependent GOGAT is favored during illumination. The isolation of fd-dependent GOGAT deficient-mutants of **A. thaliania** has led to the suggestion that fd-dependent GOGAT is only required for the photorespiratory nitrogen cycle (Somerville and Ogren 1980a). These mutant plants grow as well as the wild type plants in CO_2-enriched air (when photorespiration is suppressed) or in the dark. In fact, in high CO_2 the growth of the mutant plants is actually enhanced when compared with the growth of wild type plants in normal air. A very active NADH-dependent GOGAT activity must be present in the mutants under these enhanced growth conditions. At present, ferredoxin has not been isolated from roots, although an antigenetically similar protein has been reported (Suzuki et al. 1985). The presence of fd-dependent GOGAT conveys the notion of a metabolic role in the root. Alternatively, the presence of fd-dependent GOGAT may just represent a lack of an efficient "on-off" control for the fd-dependent GOGAT encoding gene.

Future experiments might best approach the role of fd-dependent GOGAT in the roots, by studying the flow of metabolites through the nitrogen pathway of the roots. For such experiments to be revealing, NADH-dependent GOGAT would have to be inactivated.

Genetic Analyses of GLUS Mutants

Twenty-six mutants deficient in fd-dependent GOGAT activity have been characterised in **A. thaliania**. The mutations were all allelic and recessive to the WT gene. Each mutant strain was found to be deficient only for fd-dependent GOGAT activity, NADH-dependent GOGAT activity was not affected. Thus, the presence of NADH-dependent GOGAT appears to meet the necessity for the primary assimilation of NH_4^+ for protein synthesis and growth.

Surprisingly, the photorespiratory screen for mutants yielded only **GLUS** mutants with positive CRM levels. All the mutants were obtained from the second generation of an EMS-mutagenised population. In both prokaryotes and eukaryotes, the predominant result of EMS-mutagenesis concerns single base pair changes (Coulondre and Miller 1977; Nalbantoglu et al. 1983). Null CRM mutants using EMS as a mutagen have been isolated in eukaryotes. Positive CRM mutants with altered protein products have also been isolated (Epstein et al. 1974; Nalbantoglu et al. 1983). Since the screen for mutants should have been non-selective for any step in gene expression, it was expected that null mutants (or mutants

with altered GOGAT) would be found.

The GLUS mutants examined to date are deficient for fd-dependent GOGAT activity, fd-dependent GOGAT activities on the other hand are not completely absent, nor is the fd-dependent GOGAT protein significantly altered in structure. Speculatively, the gene sequence for fd-dependent GOGAT in A. thaliania codes for another function necessary for life and morever, any alteration in the sequence is lethal. It is also possible that not enough mutants have been examined and in the future, null mutants may be found in A. thaliania. Further studies to characterise the molecular defects in the GLUS strains, await the cloning of the fd-dependent GOGAT gene.

The Effect of the GLUS Lesions on the PCR Cycle

The present studies on the activity of the PCR cycle, (as measured by determinations of RuBP levels and the assays of RuBISCO, FBPase and R5P kinase activities), have shown that this cycle was altered in GLUS plants photosynthesizing under photorespiratory conditions. The characteristic inhibition of photosynthetic CO_2 exchange under photorespiratory conditions (Somerville and Ogren 1982a) was shown to occur in the GLUS plants results studied (Fig. 14) as previously demonstrated by Somerville and Ogren (1980a). However, the data from this investigation did not confirm the presence of a lack of carbon (Somerville and Ogren

1980a; Somerville and Ogren 1983) nor was it clearly evident that there was an inactivation of RuBISCO, which might initiate the decline in photosynthetic CO_2 exchange.

The lack of a clear relationship between the RuBP levels and the inhibition of photosynthetic CO_2 exchange was surprising. Kirk and Heber (1976) convincingly demonstrated with isolated intact chloroplasts that a major withdrawal of carbon from the chloroplast in glycolate synthesis, (without any concurrent return of this carbon) essentially depletes the PCR cycle of carbon. Recently, it has been suggested that the isolation of chloroplasts from their cellular milieu prevents the proper exchange of phosphate and carbon between the chloroplast and the cytosol, possibly resulting in the abolishment of normal regulatory controls in metabolism (Dietz and Heber 1984a; Stitt et al. 1985). The finding that RuBP levels are similar between the WT and GLUS plant at a time when photosynthetic CO_2 exchange was declining indicates that something other than a complete depletion of RuBP initiates the inhibition in photosynthetic CO_2 exchange.

The activities of RuBISCO, R5P kinase and FBPase are indeed altered in GLUS plants photosynthesizing under photorespiratory conditions, supporting Creach and Stewart's (1982) proposal that the regulation of key PCR cycle enzymes is involved during the inhibition of CO_2 exchange in plants blocked in the PCO cycle. However, after 10 min of illumination in a photorespiratory atmosphere, the activity

of these enzymes in both strains, were approximately similar (Fig. 14; Tables 8 and 9). These enzymes do decline in activity between 10 and 20 min after the onset of illumination. Previously, Chastain and Ogren (1985) had shown that the RuBISCO activation state is affected in a number of photorespiratory-deficient mutants of **A. thaliania**. In some cases (for instance in the **SAT** mutant) the activation state of RuBISCO correlated approximately with photosynthetic CO_2 exchange decline. The inhibition of RuBISCO activation in other PCO cycle mutants varied, and it was stated that in **GLUS** plants the inhibition of RuBISCO lagged behind the inhibition of photosynthetic CO_2 exchange. The results described in the present study with regards to the relationship of RuBISCO activity to CO_2 exchange, support the views of Chastain and Ogren (1985). It appears that with respect to what causes the decline in RuBISCO activity, the altered metabolism of **GLUS** plants under photorespiratory conditions is different from that of the **SAT** mutant.

Two other enzymes of the PCR cycle are FBPase and R5P kinase (Lilley 1983). Both enzymes were light-activated and their activity decreased in **GLUS** plants photosynthesizing under photorespiratory conditions (Tables 8 and 9). The inhibition of enzyme activity for these two enzymes could be the result of a common mechanism or separate inhibitory interactions with stromal metabolites. As these enzymes are

regulated by a number of metabolites (Lilley 1983) the cause of their decline in activity is unclear. Future research using nonaqueous isolation of chloroplasts should concentrate on determining the levels of stromal metabolites known to have regulatory roles (Gerhardt and Heldt 1984).

Conceivably, the enzyme assays used in the present study were inadequate in accurately determining the activation state for these enzymes. It is possible that in vivo, these enzymes were inhibited by certain negative effectors, binding to the respective proteins and inhibiting the enzymes catalytically and / or their activation levels (Hatch and Jensen 1980; Badger and Lorimer 1981; Lilley 1983). Upon homogenisation and dilution of the extracts by the assay mix, these effectors were lost, and the enzyme's activity increased. Similar explanations have been offered for the varied results with regard to the actual alterations of RuBISCO activation by certain effectors (Badger and Lorimer 1981; Sefter et al. 1986). If the assays reflect the in vivo rates, then the determination of chloroplast metabolites may be essential for determining the cause for the initial decline in photosynthetic CO_2 exchange.

Previously, Somerville and Ogren (1980a) determined that NH_4^+ accumulated in the GLUS mutant, to levels purportedly high enough to uncouple photophosphorylation. The rise in NH_4^+ apparently adds a complicating factor to the interpretation of the data presented in this investigation. After 40 min of illumination, RuBISCO

activity in the GLUS strain measured only 60% of the activity of the WT strain (Fig. 15). A decrease in an enzyme's activity should result in the accumulation of its substrate, i.e. RuBP. In AAN-treated soybean leaf discs, RuBP levels are higher than in control leaves (Creach and Stewart 1982). Also, AAN inhibition of the PCO cycle inactivates RuBISCO in detached leaves of A. thaliania (Chastain and Ogren 1985). Since RuBP levels are lower in the mutant after 40 min of illumination in a photorespiratory atmosphere (Table 10), it would therefore appear that the presence of NH_4^+ in GLUS leaves uncouples photophosphorylation. However, the proposed stromal reduction of ATP may not be the only cause for RuBP decline. The amount of RuBP present in the mutant will be the result of the activity of RuBISCO, the quantity of RuBISCO protein (noncatalytic binding sites) and the level of carbon available in the PCR cycle for RuBP synthesis. The amount of carbon available for RuBP synthesis will be determined by the degree to which carbon returns from the PCO cycle (Lorimer and Andrews 1981; Bourquin and Fock 1983), the rate of carbon entrance from carbon storage pools (Ludwig and Canvin 1971; Stitt et al. 1985) and the level of ATP for substrate phosphorylation of R5P (Bassham 1965). Thus, the level of RuBP is the result of many processes, which have not been fully examined in the GLUS strain. In addition, the rates of many of the above processes are growth condition

dependent (Stitt et al. 1985). This latter phenomenon may explain the different RuBP results between PCO cycle blocked and control plants obtained in this study when compared to those reported by Creach and Stewart (198[illegible]). Certainly, this non-confirming of results is disturbing. Accordingly future studies should include a comparison of the RuBP levels of **GLYD** and **GLUS** strains of **A. thaliania** obtained from plants grown in the same growth chamber. However, the time differences of **RuBISCO** inactivation **between these** two strains reported by Chastain and Ogren (1985) and shown in the present study (Fig. 15) would suggest that the accumulation of NH_4^+ provides a unique contribution to the metabolism of **GLUS** plants.

The Effects of NH_4^+ on Metabolism of A. thaliania

The reason behind the decline of CO_2 fixation in **GLUS** plants remains unknown. Two explanations previously offered, the accumulation of NH_4^+ (Somerville and Ogren 1980a) and the build-up of glyoxylate (Cook and Tolbert 1982), were examined. In Figure 16, NH_4Cl was shown to inhibit photosynthesis in **WT A. thaliania** plants. For the **GLUS** strain, photosynthesis declined at a time when the measured NH_4^+ level was less than that required to inhibit the WT strain. These different results might be caused by an experimental artefact. The NH_4^+ in the NH_4Cl-fed plants was largely from an external source, while the NH_4^+ in the **GLUS** strain was derived from the mitochondrially localised

glycine decarboxylase reaction (Tolbert 1980). Possibly, the externally supplied NH_4^+ becomes trapped in the acidic vacuole and hence the level needed to inhibit photosynthesis is actually lower than the amounts determined in the leaves. Conversely, it is conceivable little NH_4 is trapped in the vacuole and that therefore the internally produced NH_4^+ may reach the chloroplast in a greater proportion. Nonetheless, until such a partitioning of NH_4^+ from internal versus external sources has been demonstrated, it is assumed that the differences observed in the onset of photosynthetic inhibition in the GLUS and NH_4Cl-fed WT plants represent two separate mechanisms of inhibition. The latter being an uncoupling of photophosphorylation, and the former some other cause, possibly involving the reduction of PCR cycle enzyme activities (Creach and Stewart 1982).

After 20 min of photosynthesis under photorespiratory conditions, the NH_4^+ content in the GLUS strain was rising above 2 mM (Fig. 17) which in some studies with intact chloroplasts would be high enough to lower stromal ATP levels (Tillberg et al. 1977) and uncouple photosynthetic electron transport (Slovacek and Hind 1980). Under these circumstances RuBP levels would be depleted as RuBP synthesis requires ATP (Bassham 1965). The data indicate however that the percent recovery of ^{14}C in RuBP was increased in the GLUS plant when compared to the WT strain, while the percent recovery of ^{14}C was decreased in the

NH_4Cl-fed WT plant (Table 12). It should be mentioned however that NH_4^+ was not completely effective in uncoupling photophosphorylation as some ^{14}C was recovered in RuBP. The simplest interpretation of the increased recovery of ^{14}C in RuBP in the **GLUS** strain was a reduction in RuBISCO activity. This conclusion was based on the concept that a rate limitation in a pathway results in the accumulation of the substrate. It can be assumed that RuBISCO was inactivated after 50 min of photosynthesis in 50% O_2 and 335 ppm CO_2 (remaining N_2). Chastian and Ogren (1985) reported that the RuBISCO activity was reduced after a treatment of 50 min in a similar gas regime as used for the **GLUS** mutant in the present study. The slight increase in percent recovery of ^{14}C in FBP, also suggests that FBPase activity was decreased by this time (Table 12). Earlier in the present study it was shown that these two enzymes showed a reduced activity during photosynthesis under photorespiratory conditions (Fig. 15; Tables 8 and 9). In addition the RuBP level was lower in the **GLUS** strain after 45 min of photosynthesis in 21% O_2 and 340 ppm CO_2 (see Table 10). The size of the RuBP pool is reported to be small (Jensen and Bahr 1977), and thus the 10 min labeling time with $^{14}CO_2$ was expected to saturate the RuBP pool in the experiments reported in Table 12. The causes for this discrepancy of the data between these two experiments were left unresolved, although the later experiment was done in 50% O_2 (Table 12), while the former was done in 21% O_2. Edwards and Walker (1984) have

shown that the optimum P_i level for maximum rates of photosynthesis in isolated wheat chloroplasts varies with the level of photorespiratory activity. It is possible that the level of RuBP is not critical for the decline in photosynthetic CO_2 exchange, but rather a separate process involving the P_i regulatory system as described by Heldt et al. (1978). Thus, since two different O_2 atmospheres were used, the process of inhibition may have set-in with a different time course independent of RuBP levels.

The reduced percent recovery of ^{14}C in sucrose (Table 12) constitites a similar metabolic result between the NH_4Cl-fed WT plant and the **GLUS** strain. From the literature it is known that feeding intact tissue systems NH_4^+ reduces sucrose synthesis (Platt et al. 1977; Walker et al. 1984). It is thought that in order to accomodate the increased presence of NH_4^+ the reduction in sucrose synthesis results from a carbon diversion to the TCA cycle and subsequently to the synthesis amino acid (Platt et al. 1977). In the NH_4Cl-fed WT plant, there was an increased recovery of percent ^{14}C in the amino acid fraction. In the **GLUS** plant, probably as a result of photorespiratory loss of NH_4^+, a decreased recovery of percent ^{14}C in the amino acid fraction was noted (Table 12). At present, no cogent explanation has been developed to explain the partitioning of cytoplasmic carbon in normal plants (Stitt et al. 1985). Generally however, when there is a shortage of carbon for export from the

chloroplast, sucrose synthesis is reduced (Stitt et al. 1983). Thus, the lowered recovery of ^{14}C in sucrose of the GLUS strain, might be due to carbon shortage and not increased NH_4^+.

Malate was found to be a major product of photosynthetic $^{14}CO_2$ fixation under photorespiratory conditions (Table 12). A similar result has been reported for the catalase-deficient mutant of barley (Kendall et al. 1983) and wheat leaves incubated with MSO under photorespiratory conditions (Walker et al. 1984). Malate has many functions in metabolism, such as replenishing the TCA cycle, generating the NAD(P)H and a role in the cyanide resistant respiratory pathway (Lance and Rustin 1984). The ^{14}C recovered in malate may originate from the PGA, as it has been shown that malate can be synthesized from PGA (Ruffner et al. 1983). The conversion of PGA to malate is thought to occur in the cytosol and possibly at the expense of cytosolic sucrose synthesis. How this occurs is unknown (Stitt et al. 1985). In Scenedesmus, the rapid labeling of malate by $^{14}CO_2$ is prevented for 66% to 97% by the presence of malonate (Bassham et al. 1951). Malonate is an effective inhibitor of succinate dehydrogenase and tobacco leaves treated with it accumulate succinate (Vickery and Palmer 1957). Thus, the increase in malate labeling could be via increased TCA cycle activity. At the time of the experiments, the present author was unaware of the malonate experiments.

Another explanation for the percent increase recovery of ^{14}C in malate is via PEP carboxylase activity in the leaf (Walker et al. 1984). This enzyme is present in many C_3 plants and the product of this reaction is OAA, which can be converted to malate (Lance and Rustin 1984). A direct technique, to resolve the question as to whether ^{14}C-malate arose from the carboxylation of PEP, would be to determine the intramolecular distribution of ^{14}C in malate (Levi et al. 1974). Since this technique was not available to the present author, an indirect procedure was used, whereby malate was labeled in the dark. A comparison of the subsequent metabolism in the light was carried out.

The pattern of ^{14}C distribution observed in the metabolites and recovered from labeling both WT and GLUS plants in the dark with $^{14}CO_2$, provided evidence that PEP carboxylase was active in the leaf tissue of A. thaliania. The major products recovered (malate, aspartate and citrate) after labeling in the dark are all related to the metabolism of OAA. Thus, the recovery of ^{14}C in malate can be explained by the conversion of OAA to malate as catalysed by malate dehydrogenase (Latzko and Kelly 1983). The label in citrate would be from OAA that has entered the TCA cycle. Glutamate could receive ^{14}C via the conversion 2-oxoglutarate of the TCA cycle into glutamate by an aminotransferase activity. Aspartate could also be labeled via OAA due to an aminotransferase activity. The recovery of label in the

insoluble fraction might indicate amino acid incorporation into the protein fraction.

When the plants for the WT and **GLUS** strains were illuminated, and the distribution of ^{14}C in the various metabolites determined, differences in the recovery of total ^{14}C and in the pattern of metabolites labeled were noted. The total recovery of ^{14}C was 87% of the initial amount present in the WT. For the mutant 96% of the label was recovered (Table 1[illegible]. The loss of ^{14}C was probably due to respiratory release of $^{14}CO_2$. Howev[illegible], some ^{14}C was recovered in the sugar bisphosphates (a[illegible] and neutral fractions. Similar results and conclusions have been obtained from studies in which **Chlorella** cells were supplied with ^{14}C-glucose in the dark and then incubated in the light, prior to analysis of ^{14}C metabolites (Moses et al. 1959). It appears from the data that the dark respiration was partially suppressed in the mutant. Another difference between the **GLUS** and WT plants concerns the higher recovery of label in malate (8%) in the **GLUS** strain when compared to the WT (Table 13).

Graham and Cooper (1967) reported that in similar experiments the percent recovery of ^{14}C in malate increases after illumination in mung bean. Bocher and Kluge (1978) using spinach reported that in similar experiments the percent recovery of ^{14}C in malate decreases in the light. The present data (Table 13) show that in **A. thaliania** the percent recovery of ^{14}C in malate has increased after 45 min

of photosynthesis in light. There is no obvious explanation for the difference in the data presented in these various studies.

When compared to the experiments described above (involving dark fixation of $^{14}CO_2$), a different pattern of ^{14}C incorporation in metabolites was observed when ^{14}C-malate was supplied to leaf strips in the dark and the distribution of ^{14}C metabolites recovered following illumination, was determined. After labeling during and prior to illumination, approximately 30% of the label in both GLUS and WT plants was recovered in malate (Table 14). The percentage of ^{14}C decreased during the illumination period from 34% to 19% in the WT, and from 33% to 28% in the mutant. Again, malate metabolism appears to be restricted in the mutant. The decrease in percent label recovery in malate obtained in this experiment contrasts sharply with the increase in percent label recovery in the previous dark CO_2 fixation experiment (Table 13). The opposing experimental results may reflect differences in experimental procedure. In the dark CO_2 fixation experiment, intact plants were used, whereas in the malate feeding experiment leaf strips were employed. It is possible that loss of metabolites from the leaf strips, may account for the differences.

Alternatively, MacLennon et al. (1963) have shown that in plant tissues organic acids are restricted to storage pools, which are not associated with active metabolic pools,

(such as the TCA cycle). Possibly, the ^{14}C label recovered in malate by the dark CO_2 fixation and malate feeding experiments, represent different malate pools. In support of this notion, is the observation by Lips and Beevers (1966) that when $^{14}CO_2$ or U-^{14}C-malate are fed to roots containing different malate pools, these pools are not labeled equally. At present there is a paucity of information regarding the regulation and synthesis of malate, despite its central role in metabolism. Future studies of **GLUS** metabolism should determine which pools of malate are altered in the **GLUS** plant, and consequently some knowledge may be gained with regard to carbon partitioning within the cytosol and the role of malate metabolism in photorespiratory deficient plants.

Glyoxylate Metabolism

Under photorespiratory conditions, RuBISCO activity is initially normal in **GLUS** plants but later declines (Chastian and Ogren 1985). One possible explanation for this decline is glyoxylate accumulation (Cook and Tolbert 1982). Attempts were made to address this possibility by labeling photosynthetic intermediates under steady state photosynthetic conditions thereby promoting photorespiration and the subsequent comparison of the amount of label present in glyoxylate with the rate of photosynthesis for a **GLUS** plant recovering from inhibition. No apparent correlation between ^{14}C-glyoxylate levels and rate of recovery was

found. That is to say, in the dark glyoxylate disappeared more rapidly from the cell than the capacity for photosynthesis was restored. The simplest interpretation of this result is that glyoxylate has no role in the inhibition of photosynthesis.

Genetic Analysis of the GLUS Locus

The **GLUS** mutants of **A. thaliania** were isolated in a mutagenic screening procedure developed to find plants that require CO_2-enriched air for growth (Somerville and Ogren 1982a). In the original publication concerning the **GLUS** mutant, it was shown that the strain lacked fd-dependent GOGAT activity and glutamate levels were de[illegible]ed after 10 min of photorespiration (Somerville and Ogren 1980a). Presumably, either the accumulation of NH_4^+ to toxic levels or the reduced amination of glyoxlate inhibited photosynthesis. Also, it has been suggested that the reduced glutamate levels measured in the leaves of the **GLUS** strain reduce chlorophyll content, contributing to inviability (Somerville 1984). The aim of the work presented in this section was to isolate revertants of the **GLUS** locus, which would restore either one of the above processes or reduce photorespiration in normal air.

The original mutants were isolated by EMS-mutagenesis (Somerville and Ogren 1980a), which predominately yields single-base pair changes in prokaryotes and eukaryotes

(Coulondre and Miller 1977; Nalbantoglu et al. 1983). Using the strains CS 37 and CS 66, revertants with restored fd-dependent GOGAT activity were isolated. These revertants were only phenotypic revertants since they were neither characterised at the DNA level nor mapped. Hence, it cannot be determined if the original mutation has reverted, or a second site in the DNA was altered to give a functional protein. It seems likely that the original mutagenesis yielded single-base pair changes, as the CRM level was high in these two mutants (Table 7), indicating only small changes in the protein structure. In support of this notion, a high reversion frequency was measured (Table 15). Typically, the mutation frequency in **A. thaliania** using EMS approximated 4 to 10 mutants per 1000 M_2 seeds (Koorneef and van der Ven 1980; Somerville and Ogren 1982a). The reverse mutation frequency measured in the present study was only slightly lower than a typical forward mutation rate.

The reversion frequency for the CS 254 allele was lower than for CS 37 or CS 66, and none of the revertants had fd-dependent GOGAT activity restored. The isolation of revertants without the original enzyme activity restored, suggests that the original mutation was actually two or more mutations or a deletion or addition of base pairs. Somerville and Ogren (1980a) have shown that the CS 254 allele segregates in a 3:1 phenotypic ratio in the F_2 progeny from a cross to WT. Accordingly, it can be assumed that CS 254 contains a single mutation - though it is

possible that separate but very closely linked mutations exist in the strain. It is likely that the CS 254 allele was either a small deletion or addition in the structural gene, or alternatively it may be deficient in a regulatory function, on the basis that its mutant fd-dependent GOGAT protein appeared to be of normal MW as judged by immunoprecipitation onto SDS polyacrylamide gels (Plate 5). It is generally believed that small deletion-containing mutants, revert at a much lower frequency, than that in the forward direction (Freifelder 1983).

An intriguing aspect of the work on the CS 254 allele concerns the appearance of M_2 plants which appeared to have normal levels of chlorophyll and did not flower. One explanation for this observation was that these plants may be developmental mutants. For example, an information suppressor could restore fd-dependent GOGAT activity, allowing germination, growth and chlorophyll synthesis to occur at rates above the mutant levels. But the same suppressor gene might then have a deleterious effect during the flowering developmental stage, when a different set of genes are expressed (Kamalay and Goldberg 1984). In theory, the leaf material of these plants could be tissue cultured. This would allow propagation of the tissue, allowing an assessment of fd-dependent GOGAT activity and other cellular processes. In addition growth in a tissue culture would probably not require the same developmental genes as

flowering plants employ.

The lack of revertants expressing higher than normal levels of NADH-dependent GOGAT was not unexpected. Simplistically, it might be assumed that such a mutant could result from increased promoter activity. However, in eukaryotes, promoters are complex, often containing more than one region of DNA for activity and extending over many base pairs (Cohen and Meselson 1985). Since in a eukaryotic promoter the alteration of one or a few bases has little effect on activity (Donahue and Fink 1983), the success of EMS to induce a promoter up mutation in the NADH-dependent GOGAT gene appears to be limited.

Physiology of REV11 Plants

Because in the **GLUS** strain CO_2 fixation declines to 30% of the WT rate over a 30 to 50 min perod, it is unlikely that a severe block in the PCR cycle existed. A interruption of the PCR cycle reduces CO_2 fixation to zero, as it is apparent in the **PCOA** mutant of **A. thaliania** which accumulates phosphoglycolate (Somerville and Ogre[illegible] Phosphoglycolate inhibits the triose phosphate [illegible]se reaction of the PCR cycle. The gradual decline i[illegible] strain suggests either a different mechanism of i[illegible] or a gradual occurence of a block in the PCR cycle. There is more than one substrate source for aminotransferase activity in the cell (Tolbert 1980), notably serine, a vacuolar pool of glutamate and the NADH-dependent GOGAT supplying

glutamate. The **REV11** strain was not altered with regard to the serine or glutamate pool sizes, when compared with the original mutant (Tables 20 and 21). Accordingly, the slightly increased rate of CO_2 fixation observed in the **REV11** strain cannot be accounted for by a larger source of aminotransferase substrate.

It was noted that the photosynthetic CO_2 fixation in the **GLUS** strain is able to recover from the photorespiratory caused inhibition of photosynthetic CO_2 exchange when the plants were incubated in the dark (Fig. 18). Conceivably, a net synthesis of glutamate by NADH-dependent GOGAT activity or a transport of glutamate from the vacuole to the peroxisome resulted during this recovery period. Since the **REV11** strain may be altered in one of these processes it can be expected that **REV11** will recover faster from inhibition than the **GLUS** strain. However, because of the similar pool sizes of amino acids in the light and dark the above explanation does not appear plausible. On the other hand, the amino acid contents were determined by total leaf extraction and by ^{14}C incorporation and these methods may not accurately reflect all pool activities, as has been shown in previous studies with **Chlorella** (Bassham and Kirk 1964). Also, the **in vitro** assay for fd-dependent GOGAT activity may not reflect the higher **in vivo** rate of this reaction which in turn may account for the slightly higher glutamate levels. Bigelis et al. (1981) have shown that

mutationally altered enzymes are prone to damage during isolation. Such proteins are more susceptible to protease degradation and may result in low protein levels in crude extracts. Future work with these two strains should include ^{15}N labeling, which will allow for a more thorough analysis of the kinetics and extent of NO_3 reduction and NH_4^+ assimilation.

Photosynthesis is an interplay between the capture of solar energy to form ATP and NADPH and the utilization of ATP and NADPH for the acquisition of CO_2 and other compounds. CO_2 fixation requires ATP and NADPH in a ratio of 1.5 (Bassham 1965). Due to a decrease of the activity of three key PCR cycle enzymes in **GLUS** plants, a H^+ gradient might build-up to higher than normal levels. The activity decrease would in turn cause a lowered turnover of ATP, as a result of a decrease ATPase utilization of the H^+ gradient and inhibit electron transport (Portis and McCarty 1976). When electron transport activity is limited, the photochemical apparatus becomes more susceptible to light damage (Bjorkman 1980; Powles 1984). Damage to the photochemical apparatus is often manifest within hours to days as a bleaching of the chlorophyll, depending on the plant species, growth conditions and light intensity. As an extension to the above proposal, the bleaching observed in the **GLUS** strain, may not be because of a lack of glutamate but as a result of photochemical damage caused by an excess of reducing equivalents. The response of photosynthesis to

illumination is dependent on light intensity (Bjorkman 1980). At low light intensities, the photochemical reactions are limiting and photosynthesis and intensity are linearly related. At higher light intensities, the photochemical process is less limiting. This is demonstrated by a plateau response in the photosynthetic rate, during which the rate of the enzymes in the PCR cycle, or the amount of CO_2 in the air, become limiting (Dietz and Heber 1984a). Characteristic responses of photosynthesis to light intensity have been shown to be dependent on both genotype and growth conditions (Bjorkman and Holmgren 1963; Bjorkman 1980). The response is a function of many interactive components in the leaf, such as: chlorophyll composition, electron transport carrier pool sizes, level of PCR cycle activity and gas diffusion properties of the leaf (Bjorkman 1980; Powles 1984). A unifying concept from the studies concerning adaptability to light, is the role of photoinhibition as a determinative factor. Photoinhibition is the inhibition and subsequent inactivation of the photochemical apparatus by light (Powles 1984). It is thought to be caused by an excessive absorption of light energy, without enough means to dissipate this energy by the photochemical processes. Hence a severe damage to the components of the apparatus is incurred, resulting in an altered capacity for photosynthesis (Kok 1956). This can be observed by measuring alterations in the plant's photosynthetic gas exchange at different light intensities.

In Figure 20 are the respective gas exchange curves for the **GLUS**, **REV11** and **WT** strains, after being exposed to normal air for 38 to 42 hr and in response to irradiance under non-photorespiratory conditions. Under these conditions, the photosynthetic curve of the **GLUS** and **WT** strain of **Arabidopsis** differ. In limiting light no difference was apparent in the slope, suggesting that no damage to the photochemical apparatus had yet occurred. This was unexpected, as there was a 8% decrease in chlorophyll content (Table 25) and 8-20% drop in photosynthetic electron transport rate (Table 26). However, the photosynthetic electron transport rate of the **GLUS** plants did saturate at a lower light intensity, with only a slight reduction of the intial level of RuBISCO activity on day 0 (Table 27). If a general assumption has to be made as to whether RuBISCO activity is reflective of the operative capacity of the PCR cycle, then the likely explanation for the decrease in light saturation concerns either an alteration in the photochemical apparatus or the loss of a protein regulator of the PCR cycle. The photosynthetic curve for the **REV11** strain was nearly identical to that of the **WT** strain and hence quite distinct from that of the original mutant.

At present, further research with regard to the causes of the decline in CO_2 fixation and the subsequent loss of photochemical capacity, should include the **GLUS** strain. At present, limited deductions about the effects of the **GLUS** lesion on leaf physiology can be made from the analysis of

the REV11 strain. Initially, it is important that a cogent model be developed to explain the inviability of the GLUS plants. This would require an in-depth investigation into the photochemical properties of the GLUS strain under stress. The results of the present study suggest only that photochemical damage is the cause for inviability. The light-response curve for photosynthetic CO_2 exchange and the photochemical activity of isolated thylakoids are difficult to understand since the destruction of tissue allows for the possibility of creating artefactual data. Measurements of the photochemical activity in intact leaves of the GLUS strain appears to be more meaningful. Techniques such as leaf fluorescence (Sivak and Walker 1985) or light-scattering (Dietz and Heber 1984b) should be used. With the inclusion of the data from these alternative methods, it might be possible to define what component of the GLUS leaf metabolism is susceptible to damage from growth in normal air. With availability of this knowledge a different and more direct mutagenic screen procedure could be developed. This screen would be specifically developed for the alteration of the proposed component responsible for inviability and which further physiological investigations could be carried out.

The Inhibition of Photosynthetic CO_2 Exchange in GLUS Plants

Speculatively, the cause of the decline in

photosynthetic CO_2 exchange may be a disturbance in the use of photosyntetic energy (ATP and NADPH) by alterations in the P_i levels to carbon levels and/or the P_i-regulatory mechanism, thereby inhibiting PCR cycle enzymes (Heldt et al. 1978). Without doubt, the lack of glutamate reduces the glyoxylate amination in GLUS plants and this diminishes the entrance of glycerate carbon into the PCR cycle. Since a major reaction for stromal ATP consumption is in the glycerate kinase step of the PCO cycle (Kobayashi et al. 1979a), a reduction in glycerate return might increase stromal ATP levels. A rise in ATP levels, should increase the H^+ gradient across the thylakoid membrane (Slocacek 1982) and consequently inhibit linear and cyclic electron tranpsort (Portis and McCarty 1975; Kobayashi et al. 1979b; Slovacek 1982). Any reduction in electron flow should reduce the activity of the thioredoxins required for the activation of a number of chloroplast-localised enzymes (Buchanan 1980). In addition, the presence of accumulated NH_4^+ plays a role: the photorespired-NH_3 should reach the chloroplast and penetrate the thylakoid (Crofts 1967), thereby dissipating some of the H^+ gradient. Since photosynthetic CO_2 exchange will decrease to low rates, the production of NH_4^+ also decreases and a greater H^+ gradient will be build-up. Consequently, a unique aspect of the GLUS physiology in this thesis concerns the delay in the decrease of available thioredoxins in the GLUS strain. Since no NH_4^+ is present for lowering the H^+ gradient, in the other photorespiratory-

deficient mutants of **A. thaliania**, the rapid decline in RuBISCO may be a direct result of low thioredoxin activation of RuBISCO. Hence, a more rapid inhibition of RuBISCO activation level can be observed.

The decline in **GLUS** photosynthetic CO_2 exchange might initially be caused by the disturbance in the P_i-regulatory mechanism. As ATP is a small pool, an excess of P_i should be present in the stroma. The decreased incorporation of ^{14}C from $^{14}CO_2$ into the insoluble fraction (which is mainly starch in **A. thaliania**; Somerville and Ogren 1982b), constitited evidence for this. A high P_i to PGA ratio inhibits starch synthesis (Priess 1982). The unusually high export of carbon from the **GLUS** chloroplast during photorespiration results in a high stromal P_i level, inhibiting a number of enzymes. It is possible that the enzyme assays used in this thesis were inadequate and therefore only quantification of stromal metabolites by nonaqueous techniques will resolve the question as to why photosynthesis declines in **GLUS** plants.

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