学位論文

および参考論文

有村源一郎

論文リスト

主論文

Studies on the Metabolism of Nitrogen Dioxide Nitrogen in *Arabidopsis thaliana* Plants

公表論文

1. Arimura, G., Banjo, H., Irifune, K., Pwee, K.-H., Takahashi M. and Morikawa, H. (1998) Expression of the β-glucuronidase gene introduced into intact leaves attached to *Arabidopsis thaliana* plants by particle gun. *Plant Biotech*. 15: 109-111.
2. Arimura, G., Takahashi, M. and Morikawa, H. (1998) Manipulation of genes for nitrogen metabolism in plants. *In* Air Pollution and Biotechnology in Plants. Edited by K. Oomasa et al., Springer-Verlag (in press).

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1. Kawamura, Y., Takahashi, M., Arimura, G., Isayama, T., Irifune, K., Goshima, N. and Morikawa, H. (1996) Determination of levels of NO_3^-, NO_2^-, and NH_4^+ ions in leaves of various plants by capillary electrophoresis. *Plant Cell Physiol*. 37: 878-880.
2. Arimura, G., Takahashi, M., Goshima, N. and Morikawa, H. (1998) Metabolic fate of the nitrogen of nitrogen dioxide in Arabidopsis thaliana leaves. Proceedings of the XIth International Congress on Photosynthesis, Kluwer Academic Publishers (in press).
3. Arimura, G., Goshima, N., Takahashi, M., Fujita, K., Sakurai, N. and Morikawa, H. Metabolic fate of nitrogen dioxide nitrogen in plant leaves differs from that of nitrate nitrogen (submitted).
4. Arimura, G., Kawamura, Y., Banjyo, H., Irifune, K., Goshima, N. and Morikawa, H. Characterization of responses of *Arabidopsis thaliana* plants that were bombarded with an antisense *gs* cDNA followed by fumigation with nitrogen dioxide gas (submitted).

主論文

CONTENTS

INTRODUCTION

Vegetation is recognized as a sink for atmospheric pollutants, and efforts have been made to characterize the interactions between plants and pollutants. Studies of pollutant sorption by plants furnish information that can help in assessing the efficacy of atmospheric scrubbing and in understanding the fate of pollutant molecules within plants. Some of these moleculars can be phytotoxic and some can serve as plant nutrients (for example, S and N pollutants). Nitrogen dioxide (NO_2), a major air pollutant, might conceivably serve in either role (Wellburn 1990). NO_2 is known to incorporate into plants through their stomata (Saxe 1986, Okano et al. 1989, Thoene et al. 1991), and in aqueous solution (e.g., in the apoplastic water around leaf cells) NO_2 is thought to react with water and this feature increases significantly the apparent solubility of NO_2. Reaction of NO_2 with water is not just simple hydration producing nitric acid (HNO_3). Using data from conductivity experiments, Lee and Schwartz (1981) concluded that NO_2 undergoes a comparatively slow heterogeneous reaction with water to form dissolved NO_2 species which then reacts with itself to give both HNO_3 and nitrous acid (HNO_2). Over pH ranges that are biologically important, HNO_3 (pKa -1.4) and HNO_2 (pKa -3.3) ionize to nitrate and nitrite, respectively. Then those ions are converted to organic compounds such as amino acids or proteins (Yoneyama and Sasakawa 1979, Kaji et al. 1980, Lee and Schwartz 1981, Wellburn 1990, Nussbaum et al. 1993) through the nitrate assimilation pathway. Formation of ^{15}N-labeled nitrate, nitrite, and amino acids in plants fumigated with ^{15}N-labeled NO_2 has been reported (Yoneyama and Sasakawa 1979, Kaji et al. 1980), and since then it has been assumed that NO_2 nitrogen is metabolized as similar to that of nitrate in plant cells. Whether a stoichiometric relationship exists between the total nitrogen (N) derived from NO_2 and the sum of reduced and nitrate/nitrite N has not, however, been studied.

In higher plants, nitrate and nitrite ions generated from NO_2 are generally thought to be reduced by nitrate reductase (NR; EC 1.6.6.1) and nitrite reductase (NiR; 1.7.7.1) in their cytosol and chloroplast, respectively. Glutamine synthetase (GS; EC 6.3.1.2) in conjunction with glutamate synthetase (GOGAT; EC 2.6.1.53) is thought to be important for a conversion of the reduced NO_2 nitrogen to the organic form. GS catalyzes the ATP-dependent condensation of ammonia, the ultimate inorganic form of nitrogen available to the plants, with glutamate to yield glutamine (Miflin and Lea 1980, Lea 1993). Higher plant GS is an octomatic enzyme of 350-400 kDa (Lea 1993) which occurs as a number of isoenzymes, the subunits of which are encoded by a small multigene family (Tingey et al. 1987, Tingey et al. 1988, Sakamoto et al. 1989, Peterman and Goodman 1991, Li et al. 1993). These GS isoforms are located either in cytosol (GS1) or chloroplast/plastid (GS2) and in different plant organs, and assimilate ammonia derived by different physiological processes (Edwards et al. 1990, Cullimore and Bennett 1992, Lam et al. 1995). In roots, GS1 assimilates NH_3 released by breakdown of nitrogenous resources during germination (Walker and Coruzzi 1989, Peterman and Goodman 1991, Watanabe et al. 1994). In leaves, NH_3 produced from photorespiration is assimilated by GS2 (Wallsgrove et al. 1987, Edwards and Coruzzi 1989, Häusler et al. 1994). GS1 in stems and leaves is located primarily in the vascular system (Carvalho et al. 1992, Kamachi et al. 1992, Marsolier et al. 1995) and it is postulated that it functions to generate glutamine for nitrogen transport (Edwards et al. 1990, Kamachi et al. 1991). In root nodules, the primary function of the GS is the rapid assimilation of NH_3 excreted into the plant cytosol of infected cells by nitrogen-fixing bacteroids (Cullimore and Bennett 1988, Lea 1993).

As for the regulation of GS gene, induction by nitrogen compounds has been studied extensively. The gene expression of GS1 is known to response to nitrate and ammonia (Hirel

et al. 1987, Kozaki et al. 1991, Miao et al. 1991, Stanford et al. 1993, Sukanya et al. 1994, Sakakibara et al. 1996), and recently Watanabe et al. (1997) suggested that the GS1 transcript level was regulated in response to the ratio of cellular glutamine to glutamate, but not to the glutamine level alone in radish cultured cells. The other isoform (GS2) is well characterized in various plant species as light regulation of the expression (Edwards and Coruzzi 1989, Peterman and Goodman 1991) and also was regulated by ammonia in rice (Kozaki et al. 1992).

In chapter I, I will describe the results of the sequence analysis of two cDNA and genomic DNA clones for *gsR1* (GS1) and *gsL1* (GS2) isolated from the cDNA library or genomic DNA library of *Arabidopsis thaliana*, respectively.

In chapter II, I will describe the results of Northern blot analysis for GS1 and GS2 mRNA in *Arabidopsis thaliana* plants fumigated with 4 ppm NO_2. I will show that their accumulations changed in different manner in response to NO_2-fumigation in leaves.

In chapter III, I will show here an *in planta* transient expression assay using particle gun device, in which transgenes are introduced into the cells of leaves that are attached to an intact plant. Using this method, a plasmid pSG containing 663-bp fragment of cDNA of the gene encoding GS in an antisense orientation under the control of cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthetase (NOS) polyadenylation signal was bombarded into the leaves of *Arabidopsis thaliana* attached to intact plants. I will show the results of subsequent analyses including enzyme activity analysis, Western blot analysis and ion content analysis.

In chapter IV, I will describe the transgenic *Arabidopsis thaliana* plants bombarded with a plasmid that GS1 or GS2 cDNA in a sense or antisense orientation is under the control of

CaMV 35S promoter and NOS polyadenylation signal. I will show the results of the determination of GS enzymatic activity and Western blot analysis in leaves of transgenic lines.

In chapter V, I will describe about the fate of NO_2 nitrogen taken up in plant leaves. I studied there by using ^{15}N-labeled NO_2 ($^{15}NO_2$) and potassium nitrate ($K^{15}NO_3$) to clarify whether the total ^{15}N taken up in the leaves of *Arabidopsis thaliana* plants is equal to the amount of reduced ^{15}N plus nitrate/nitrite ^{15}N. I found that the metabolic fate of NO_2 nitrogen in the leaves differs from that of nitrate.

CHAPTER I

Nucleotide sequences of cDNAs and genomic DNAs for cytosolic and chloroplastic glutamine synthetse from *Arabidopsis thaliana* plants

Glutamine synthetase (GS; EC 6.3.1.2) is a key enzyme in the assimilation of ammonia, catalyzing the ATP-dependent condensation of ammonia with glutamate to yield glutamine (Miflin and Lea 1980, Lea 1993). In higher plants GS occurs as a number of isoenzymes and is encoded by a small multigene family, whose members exhibit organ specific patterns of expression (Tingey et al. 1987, Peterman and Goodman 1991, Li et al. 1993, Marsolier et al. 1995). Based on the subcellular location, GS can broadly categorized as cytosolic GS1 and chloroplastic GS2, which respectively is present in the vascular system or in mesophyll cells in plant leaves (Carvalho et al. 1992, Cock et al. 1992, Kamachi et al. 1992, Lam et al. 1995). So, the different isozymes of GS assimilate ammonia produced by different physiological processes (Edwards et al. 1990, Cullimore and Bennett 1992, Lam et al. 1995), e.g., symbiotic N_2 fixation, the reduction of nitrate or nitrite, photorespiration, amino acid catabolism (Lea 1993), or NO_2-metabolism (Kaji et al. 1980, Wellburn 1990) and probably have different physiological requirements for optimal activity (Cullimore and Bennett 1988, Temple et al. 1996). Peterman and Goodman (1991) has already isolated four GS cDNA clones for *Arabidopsis thaliana* ecotype Columbia, in which it appear to possess a single nuclear gene for chloroplastic GS2 (*gsL1*) and at least three genes for cytosolic GS1 (*gsR1*, *gsR2*, and *gsKb6*) and they diferentially express in the root, leaf, and seed organs.

In this chapter, I describe the results of the sequence analysis of two cDNA and genomic DNA clones for *gsR1* (GS1) and *gsL1* (GS2) isolated from the cDNA library or genomic

DNA library of *Arabidopsis thaliana*, respectively.

MATERIALS AND METHODS

RT-PCR

Total RNA (1 μg) extracted from *Arabidopsis thaliana* leaves was reverse-transcribed by reverse transcriptase (Superscript II; Gibco BRL, MD, U.S.A.) and a primer (5'GACTTATCCTAAGACATTGC-3' or 5'-TTGAGAGACCACATAGACA-3') which were, respectively, corresponding to nucleotide 1209 to 1228 and 1401 to 1420 in the sequences of *gsR1* (GS1) and *gsL1* (GS2) cDNAs reported by Peterman and Goodman (1991). The first-strand cDNA was amplified by adding Taq DNA polymerase (Takara Shuzo Co., Ltd., Otsu, Japan) and further primer (5'-CTGTTACTATCATCCAAACC-3' or 5'-TTCAAGTCCCAGGGATCATC-3'), which respectively correspond to nucleotide 20 to 39 in GS1 cDNA and 46 to 65 in GS2 cDNA in that report. Thirty-five cycle of amplification were performed (1 min at 95°C for denaturation; 2 min at 55°C for annealing; and 1 min at 72°C for extension).

Screening of Arabidopsis thaliana *GS1 and GS2 cDNA clones*

A cDNA library was constructed in the Lambda ZAP II vector (Stratagene, La Jolla, CA, U.S.A.) using poly(A)$^+$ RNA from leaves and stems of *Arabidopsis thaliana* ecotype C24 fumigated with 4 ppm NO_2 for 1 h, as a template. Both GS1 and GS2 cDNA clones were screened from this library (about 2×10^6 pfu, respectively) according to plaque hybridization technique (Maniatis et al. 1982) using each cDNA amplified by RT-PCR (see above), as a probe. Full-length or near full-length representatives of each class in the positive plaques

obtained in the first round of screening were initially identified by PCR, and purified by additional round of screening. The positive GS1 and GS2 cDNA clones gained were named as pGSR1 and pGSL1, respectively.

Screening of Arabidopsis thaliana *GS1 and GS2 genomic DNA clones*

A genomic library of *Arabidopsis thaliana* ecotype Columbia (6×10^5 recombinant plaques; Clontech) constructed with the λEMBL3 vector was screened by plaque hybridization with full-length or near full-length GS1 or GS2 cDNA (see above) as a probe. The representatives of each class in the positive plaques obtained in the first round of screening were purified by additional round of screening.

DNA sequence analysis

The pBluescript II SK(-) plasmids carrying the putative GS cDNA were *in vivo* excised from the λZAP II vector according to the protocol described by Stratagene. λ DNA carrying the putative GS1 or GS2 genomic DNA was digested with *Bam* HI and *Hind* III or *Bam* HI, *Xba* I and *Eco* RV, respectively, and each DNA fragment was subcloned to pBluescript II KS(+). A series of nested dilations from both ends was generated for each clone using Exonuclease III according to Henikoff (1987). The DNA sequence of each dilation was determined by the dideoxy method using DNA sequencing kit Dye Primer Cycle Sequencing Ready Reaction M13 Reverse/-21M13 (Perkin Elmer Applied Biosystems Co., Ltd., Foster City, CA, U.S.A.) in full for both strands of each clone. Sequence analysis was performed using the DNA Sequencing Software version 2.1.2 (Perkin Elmer Applied Biosystems Co., Ltd., Foster City, CA, U.S.A.).

RESULTS

Sequence analysis of GS1 and GS2 cDNA

Fig. 1A and 1B show the nucleotide sequences and deduced amino acid sequences of full-length or near full-length *gsR1* (GS1) and *gsL1* (GS2) cDNA clones from *Arabidopsis thaliana* ecotype C24. Each nucleotide sequence data reported will appear in DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AB006029 and AB006030, respectively. The GS1 cDNA clone contained a 1352 nucleotide insert. The first ATG of the sequence, the putative initiation codon, preceded an ORF of 1068 nucleotides. This ORF translates into a 356 amino acid protein and only three replaced amino acid was found, compared with that for ecotype Columbia reported by Peterman and Goodman (1991). Five kinds of the regions conserved in both prokaryotic and eukaryotic GS were also found in its ORF, which are associated with the GS active site (Rawlings et al., 1987). In those regions, ATP-binding site was in the amino acid positions from 213 to 235 and glutamate-binding site also located in amino acid positions from 289 to 298.

The GS2 cDNA clone contained a 1538 nucleotide insert. The first ATG of the sequence, the putative initiation codon, preceded an ORF of 1290 nucleotides. This ORF translates into a 430 amino acid protein and no replaced amino acid was found, compared with that for ecotype Columbia reported previously. This putative ORF also contained five conserved regions, in which ATP-binding site located in the amino acid positions from 291 to 312 and glutamate-binding site also located in amino acid positions from 367 to 376.

Sequence analysis of GS1 and GS2 genes

A genomic library of *Arabidopsis thaliana* ecotype Columbia was screened with GS1 or GS2

cDNA as a probe and two or three of the positive phage clones were isolated, respectively. Each a representative containing GS1 or GS2 gene was subcloned into pBluescript II vector and its sequence was determined in full for both stands of each clone. The nucleotide sequence of the *gsR1* (GS1) or *gsL1* (GS2) cDNA (Peterman and Goodman, 1991) was identical to each of corresponding genomic clone, with the exception of the 5' and 3' franking regions, and intron region of these genes (Fig. 2). Each nucleotide sequence data reported will appear in DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AB006031 and AB015045, respectively.

A GS1 clone contained 5314 nucleotides, including 9 exons and 8 introns; the introns began with nucleotides AG and ended with GT. A putative TATA box was found in 52 nucleotide upstream from the N-terminal end of the cDNA. On the other hand, a GS2 clone contained 7498 nucleotides, including 11 exons in which exon 1 was found in this 5' untranslated region of the cDNA and 10 introns beginning also with nucleotides AG and ended with GT. A putative TATA box was found in 64-nucleotide-upstream from the N-terminal end of the cDNA.

```
                                                                CTC      3
GTGCGTTTGATTCCATTTTTATTACTGTTACTATCATCCAAACCCTTGGTATTTGTAGCC     63
ATGAGTCTTGTTTCAGATCTCATCAACCTTAACCTCTCAGACTCCACTGACAAAATCATT    123
 M  S  L  V  S  D  L  I  N  L  N  L  S  D  S  T  D  K  I  I
GCTGAATACATATGGGTTGGTGGTTCTGGAATGGACATGAGAAGCAAAGCCAGGACTCTA    183
 A  E  Y  I  W  V  G  G  S  G  M  D  M  R  S  K  A  R  T  L
CCTGGACCAGTGACTGACCCTTCGCAGCTACCAAAGTGGAACTATGATGGTTCAAGCACA    243
 P  G  P  V  T  D  P  S  Q  L  P  K  W  N  Y  D  G  S  S  T
GGACAAGCTCCTGGTGAAGACAGTGAAGTCATCTTATACCCTCAAGCCATATTCAAAGAT    303
 G  Q  A  P  G  E  D  S  E  V  I  L  Y  P  Q  A  I  F  K  D
CCTTTCCGTAGAGGAAACAACATTCTTGTCATGTGCGATGCGTACACTCCCGCGGGTGAA    363
 P  F  R  R  G  N  N  I  L  V  M  C  D  A  Y  T  P  A  G  E
CCAATCCCGACTAACAAAAGACACGCTGCGGCTAAGGTCTTTAGCAACCCTGATGTTGCA    423
 P  I  P  T  N  K  R  H  A  A  A  K  V  F  S  N  P  D  V  A
GCTGAAGTGCCATGGTATGGTATTGAGCAAGAATACACTTTACTCCAGAAAGATGTGAAG    483
 A  E  V  P  W  Y  G  I  E  Q  E  Y  T  L  L  Q  K  D  V  K
TGGCCTGTTGGTTGGCCTATTGGTGGTTATCCCGGCCCTCAGGGACCGTACTATTGCGGT    543
 W  P  V  G  W  P  I  G  G  Y  P  G  P  Q  G  P  Y  Y  C  G
ATTGGAGCAGACAAATCTTTTGGCAGAGATGTTGTTGATTCTCACTACAAGGCCTGCTTA    603
 I  G  A  D  K  S  F  G  R  D  V  V  D  S  H  Y  K  A  C  L
TACGCTGGGATCAACATTAGTGTCATCAATGGAGAAGTCATGCCGGGTCAGTGGGAGTTC    663
 Y  A  G  I  N  I  S  V  I  N  G  E  V  M  P  G  Q  W  E  F
CAGGTCGGTCCAGCTGTTGGTATCTCGGCCGCTGATGAAATTTGGGTCGCTCGTTACATT    723
 Q  V  G  P  A  V  G  I  S  A  A  D  E  I  W  V  A  R  Y  I
TTGGAGAGGATCACAGAGATTGCTGGTGTAGTGGTATCTTTTGACCCGAAACCGATTCCC    783
 L  E  R  I  T  E  I  A  G  V  V  V  S  F  D  P  K  P  I  P
GGTGACTGGAACGGTGCTGGTGCTCACTGCAACTACAGTACCAAGTCAATGAGGGAAGAA    843
 G  D  W  N  G  A  G  A  H  C  N  Y  S  T  K  S  M  R  E  E
GGCGGTTACGAGATCATCAAGAAAGCAATCGATAAATTGGGACTGAGACACAAAGAACAC    903
 G  G  Y  E  I  I  K  K  A  I  D  K  L  G  L  R  H  K  E  H
ATTGCTGCTTACGGTGAAGGCAATGAGCGTCGTCTCACCGGTCACCACGAGACTGCTGAC    963
 I  A  A  Y  G  E  G  N  E  R  R  L  T  G  H  H  E  T  A  D
ATCAACACTTTCCTTTGGGGTGTTGCGAACCGTGGAGCATCGATCCGAGTAGGACGTGAT   1023
 I  N  T  F  L  W  G  V  A  N  R  G  A  S  I  R  V  G  R  D
ACTGAGAAAGAAGGGAAAGGATACTTTGAGGATAGGAGGCCAGCTTCGAACATGGATCCT   1083
 T  E  K  E  G  K  G  Y  F  E  D  R  R  P  A  S  N  M  D  P
TACATTGTCACTTCCATGATTGCAGAGACTACAATCCTCTGGAATCCTTGATGATCATCA   1143
 Y  I  V  T  S  M  I  A  E  T  T  I  L  W  N  P  *
GATCAAGAAAAAATCTTGAATGTCACTCAAATTTGTGTTTCTTGCAAGATTCAAAGTTTG   1203
TGTTCTCTATCAAGCAATGTCTTAGGATAAGTCAAAGATTTGCTCTGCTTATTCTGCTTT   1263
TTATTTACTTCACATCCTATTGAAAACATTTCTGTGTATTATTTATGAATAAACATTATC   1323
TTAAAAAAAAAAAAAAAAAAA
```

■ :ATP-binding site
■ :Glu-binding site
■ :Other conserved region

Fig. 1A Nucleotide and deduced amino acid sequences of the *gsR1* (GS1) cDNA for *Arabidopsis thaliana* ecotype C24. The replaced amino acid compared with *Arabidopsis thaliana* ecotype Columbia is green-colored and each color box shows the conserved region of GS gene for some species. Asterisk indicate the stop codon.

```
                                                        GCCATCTGTTACAAACA    17
CCAAACTCTCCTGATTCATCAGTTTTAAGTCTTCTTCAAGTCCCAGGGATCATCAATCCA    77
ATGGCTCAGATCTTAGCAGCTTCTCCAACATGTCAGATGAGAGTGCCTAAACACTCATCA   137
 M  A  Q  I  L  A  A  S  P  T  C  Q  M  R  V  P  K  H  S  S
GTCATTGCATCATCATCCAAGTTATGGAGCTCTGTTGTGTTGAAACAGAAGAAGCAGAGC   197
 V  I  A  S  S  S  K  L  W  S  S  V  V  L  K  Q  K  K  Q  S
AACAACAAAGTCAGAGGCTTTAGAGTTCTTGCTCTCCAATCTGATAACAGTACTGTCAAT   257
 N  N  K  V  R  G  F  R  V  L  A  L  Q  S  D  N  S  T  V  N
AGAGTTGAGACTCTTCTCAATTTAGACACCAAACCTTACTCTGACAGGATCATTGCTGAA   317
 R  V  E  T  L  L  N  L  D  T  K  P  Y  S  D  R  I  I  A  E
TACATTTGGATTGGAGGATCTGGAATTGACCTTAGAAGCAAGTCAAGGACTATCGAAAAG   377
 Y  I  W  I  G  G  S  G  I  D  L  R  S  K  S  R  T  I  E  K
CCGGTGGAGGATCCTTCTGAGCTACCTAAGTGGAACTACGATGGTTCGAGTACCGGTCAA   437
 P  V  E  D  P  S  E  L  P  K  W  N  Y  D  G  S  S  T  G  Q
GCACCTGGTGAAGATAGTGAAGTGATTCTATACCCGCAAGCTATCTTCAGAGATCCTTTC   497
 A  P  G  E  D  S  E  V  I  L  Y  P  Q  A  I  F  R  D  P  F
CGTGGAGGCAATAACATCTTGGTTATCTGTGATACTTGGACACCAGCTGGTGAGCCAATT   557
 R  G  G  N  N  I  L  V  I  C  D  T  W  T  P  A  G  E  P  I
CCAACAAACAAACGTGCTAAAGCTGCTGAGATCTTCAGTAACAAGAAGGTCTCTGGCGAG   617
 P  T  N  K  R  A  K  A  A  E  I  F  S  N  K  K  V  S  G  E
GTTCCATGGTTCGGCATTGAACAAGAGTACACTTTACTTCAGCAAAACGTCAAATGGCCT   677
 V  P  W  F  G  I  E  Q  E  Y  T  L  L  Q  Q  N  V  K  W  P
TTAGGTTGGCCTGTTGGAGCGTTCCCTGGTCCTCAGGGTCCTTACTACTGTGGAGTTGGA   737
 L  G  W  P  V  G  A  F  P  G  P  Q  G  P  Y  Y  C  G  V  G
GCTGACAAGATTTGGGGGCGTGACATTTCAGATGCTCATTACAAAGCTTGTTTATATGCT   797
 A  D  K  I  W  G  R  D  I  S  D  A  H  Y  K  A  C  L  Y  A
GGAATTAACATTAGTGGTACTAATGGTGAAGTTATGCCTGGACAGTGGGAGTTCCAAGTT   857
 G  I  N  I  S  G  T  N  G  E  V  M  P  G  Q  W  E  F  Q  V
GGCCCGAGCGTAGGAATCGATGCAGGTGATCATGTTTGGTGTGCTAGATACCTTCTTGAG   917
 G  P  S  V  G  I  D  A  G  D  H  V  W  C  A  R  Y  L  L  E
AGAATCACAGAACAAGCTGGTGTTGTCCTAACACTTGATCCCAAACCGATAGAGGGAGAC   977
 R  I  T  E  Q  A  G  V  V  L  T  L  D  P  K  P  I  E  G  D
TGGAACGGTGCTGGTTGCCACACCAATTACAGTACAAAGAGCATGAGAGAGGAAGGAGGA  1037
 W  N  G  A  G  C  H  T  N  Y  S  T  K  S  M  R  E  E  G  G
TTTGAGGTGATCAAGAAGGCTATCTTGAACCTCTCACTTCGCCACAAGGAGCACATCAGT  1097
 F  E  V  I  K  K  A  I  L  N  L  S  L  R  H  K  E  H  I  S
GCCTACGGTGAAGGAAACGAGAGAAGGTTGACCGGAAAGCACGAGACAGCTAGTATTGAC  1157
 A  Y  G  E  G  N  E  R  R  L  T  G  K  H  E  T  A  S  I  D
CAGTTCTCATGGGGCGTGGCTAACCGTGGATGCTCTATTCGTGTGGGACGTGACACCGAG  1217
 Q  F  S  W  G  V  A  N  R  G  C  S  I  R  V  G  R  D  T  E
GCGAAAGGAAAAGGTTACTTAGAAGATCGCCGTCCAGCATCTAACATGGACCCATACATT  1277
 A  K  G  K  G  Y  L  E  D  R  R  P  A  S  N  M  D  P  Y  I
GTGACCTCACTTTTGGCAGAGACCACACTCCTGTGGGAGCCAACTCTTGAGGCTGAAGCC  1337
 V  T  S  L  L  A  E  T  T  L  L  W  E  P  T  L  E  A  E  A
CTTGCAGCTCAAAAGCTTTCTTTGAATGTTTAAAATTAGTCGAAACTTTCATGAATCTGA  1397
 L  A  A  Q  K  L  S  L  N  V  *
TGAACACACGTGTCTATGTGGTCTCTCAAGTTGTTTAAACATTCGGATTAAGACATTGTT  1457
TGTTGTCTTTTCATTTGCATTTTTAAAACTCAGAATTGTATGGACAATGTTCATCCTTTT  1517
ATATTGGTTCTTTTGACTGTTAAAAAAAAAAAAAAAAA
```

■ :ATP-binding site

■ :Glu-binding site

■ :Other conserved region

Fig. 1B Nucleotide sequence and deduced amino acid sequence of the *gsL1* (GS2) cDNA for *Arabidopsis thaliana* ecotype C24. Each color box shows the conserved region of GS gene for some species and asterisk indicate the stop codon.

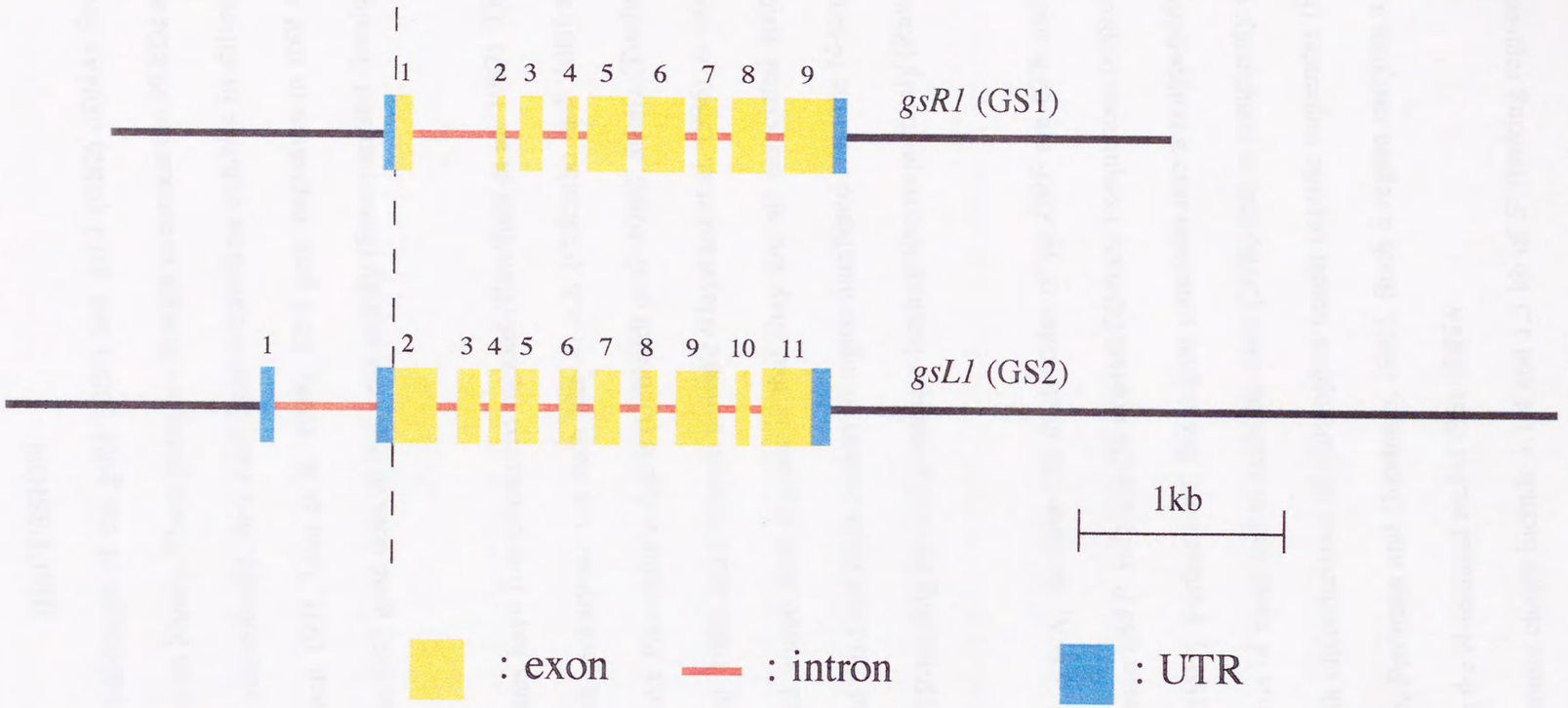


Fig. 2 Structures of GS1 and GS2 genes for *Arabidopsis thaliana*

DISCUSSION

I isolated full-length or near full-length of the *gsR1* (GS1) and *gsL1* (GS2) cDNAs from *Arabidopsis thaliana* leaf and stem library. These genes are thought to encode the 39 kDa and 43 kDa of GS polypeptides, respectively, and each gene expression exhibits in different tissues (Peterman and Goodman 1991, Lam et al. 1995). GS1 gene expresses in root and germinated seed cells, whereas GS2 gene does in leaf cells mainly (Peterman and Goodman 1991).

Both these GS cDNA clones have five conserved regions (Rawlings et al. 1987). Those regions are conserved in both prokaryotic GS such like *E. coli* (Colombo and Villafranca 1986) and eukaryotic GS as like *Phaseolus vulgaris* (Gebhardt et al. 1986), alfalfa (Tischer et al. 1986), rice (Sakamoto et al. 1989) and Chinease hamster (Hayward et al. 1986). A striking feature about five conserved amino acid regions is that they are all associated with the proposed GS active sites, and third and forth conserved region numbered from the N-terminal end are deduced to be the ATP-binding site and glutamate-binding site, respectively (Rawlings et al. 1987).

In the sequence of GS2 cDNA, the first 153 nucleotides of its ORF are 62% and 60% similar to that of the *P. sativum* and *P. vulgaris* chloroplast GS transit sequences, respectively (Peterman and Goodman, 1991). Furthermore, this region translates into a polypeptide with an unusually high proportion of basic amino acids residues (20%) that is completely devoid of acidic amino acids, both characteristics of chloroplast transit peptide sequences (Karlin-Neumann and Tobin 1986, Peterman and Goodman, 1991). Such a region carrying a transit sequence was not found in the N-terminal end of GS1 cDNA.

The GS1 and GS2 genomic clones include a 1.4 and 1.3 kb of 5' flanking regions of the

genes, respectively. In those regions, respectively, the 17 and 14 binding sites for NIT-2 protein factors were found (Table 1), which is the major regulatory factor of nitrogen metabolism in fungi (Fu and Maraluf 1990, Marzluf 1993). The sequence motifs have widely found in the 5' flanking regions of genes for *Arabidopsis thaliana* NR and NiR, rice FNR, and maize Fd (Matsumura et al. 1997), which are known as nitrate responsible genes. The transcripts of GS and GOGAT genes are also thought to be significantly affected by cellular nitrogen status (Kozaki et al. 1991, Watanabe et al. 1996, Watanabe et al. 1997), though little is known about it. Table 1 shows various length of the 5' flanking regions of GS1, GS2, and NADH-GOGAT genes from *Arabidopsis thaliana*, *Medicago sativa* L., *Phaseolus vulgaris* L., *Pisum sativum* L., and *Oryza sativa* L. also exhibit some numbers of the NIT-2 motifs. We also analyzed the putative sequence motifs in their 5' flanking regions, which are necessary for nitrate-dependent transcription of *Arabidopsis thaliana* NR genes and conserved in the 5' flanking regions of the other nitrate-inducible plant genes [A(C/G)TCA], suggested by Hwang et al. (1997), and consequently some numbers of the motifs were found in their regions for GS and GOGAT genes from some kinds of plants (Table 1). The presences of these sequence motifs in the 5' upstream regions of their genes suggest that cellular nitrogen status affects the expressions of GS/GOGAT genes through the trans-regulatory factors as similar as those of the NR and NiR genes, though it is not clear yet about the presence of certain factors except for the NIT-2, and the direct nitrogenous signal(s) which affects their gene expressions, e.g., nitrate, ammonia, glutamine, and/or glutamate.

Table 1 The presences of the sequence motifs in the 5' flanking regions of GS and GOGAT genes

Gene	Length of 5' upstream region (bp)	No. of binding [a] sites for NIT-2	No. of the similar motifs necessary [b] for nitrate-dependent transcription	Reference
A. thaliana GS1	1394	17	6	This study
A. thaliana GS2	1253	14	3	This study
M. sativa GS1	620	2	1	Tischer et al. (1986)
P. sativum GS1 (GS3A)	1833	25	3	Walker et al. (1995)
P. sativum GS1 (GS3B)	1140	14	1	Walker et al. (1995)
P. vulgaris GS2	3006	16	5	Cock et al. (1992)
M. sativa NADH-GOGAT	2333	21	2	Vance et al. (1995)
O.sativa NADH-GOGAT	3700	22	4	Unpublished

[a] Putative binding sites for NIT-2 [TATC(TAG)(ACT) or (AGT)(TCA)GATA].

[b] Similar sequence motifs necessary for nitrate-dependent transcription of *Arabidopsis thaliana* NR genes and conserved in the 5' flanking regions of other nitrate-inducible plant genes [A(C/G)TCA] (Hwang et al. 1997).

CHAPTER II

Expression of GS genes in response to fumigation with nitrogen dioxide

Plants are reported (Rogers et al. 1979, Yoneyama and Sasakawa 1979, Wellburn 1990) to assimilate the nitrogen in nitrogen dioxide (NO_2) to organic compounds, including amino acids in their leaves (Kaji et al. 1980). GS is known to be a key enzyme to incorporate ammonia derived from NO_2 to glutamine. However, the response of GS isozymes to NO_2 in the levels of mRNA and enzyme activity have not been cleared yet.

In this chapter, I show the results of Northern blot analysis for *gsR1* (GS1) and *gsL1* (GS2) mRNAs in leaves of *Arabidopsis thaliana* fumigated with 4 ppm NO_2. I show that their accumulations changed differntially for up to 10 h after the start of NO_2-fumigation.

MATERIALS AND METHODS

Plant material

Arabidopsis thaliana ecotype C24 plants were sown in vermiculite and perlite (1:1, v/v) in plastic pots, and the pots placed in a growth chamber (model ER-20-A; Nippon Medical & Chemical Instruments Co., Osaka, Japan). After germination, seedlings were grown under continuous light (70 μE $m^{-2}s^{-1}$) at 22.0±0.3°C at a relative humidity of 70±4% and irrigated at 4-day intervals with a half-strength solution of the inorganic salts of Murashige and Skoog's medium (Murashige and Skoog 1962) that contained 19.7 mM nitrate and 10.3 mM ammonium salts.

Fumigation with NO_2

Six-week-old *Arabidopsis thaliana* seedlings in the plastic pots were placed in a fumigation chamber (NC-1000-SC, Nippon Medical & Chemical Instruments Co., Osaka, Japan) and treated with 4.0±0.3 ppm NO_2 for up to 10 h under continuous light (70 μE m^{-2} s^{-1}), 340±80 ppm CO_2 at 22.0±0.3°C, and a relative humidity of 70±4%. The pots were covered with polyethylene bags to avoid dissolution of NO_2 into the soil layer.

PCR amplification

Each PCR amplification of the 3'UTR fragment of *gsR1* (GS1) or *gsL1* (GS2) cDNA was performed with plasmid DNA pGSR1 and pGSL1 (see Chapter I) as a template, respectively. The primer sequences used for amplifications of the cDNA fragments were, respectively, 5'-CCGGATCCGATGATCATCAGATC-3' (sense) and 5'-CCGAATTCCAATAGGATGTGAAG-3' (antisense) for amplification of a fragment carrying 153 bp of GS1 cDNA, and 5'-CCCGAATTCGAATCTGATGAACACACGTG-3' (sense) and 5'-CCCCTCGAGCGTTTGGACATGCTCTAACA-3' (antisense) for amplification of a fragment carrying 164 bp of GS2 cDNA. Thirty-five cycles of amplification, each of which consisted of 1 min at 95°C for denaturation, 2 min at 60°C for annealing, and 1 min at 72°C for extension, were made. Each amplified cDNA fragment was used for subsequent Northern blot analysis as a specific probe.

Northern blot analysis

Leaves were harvested 0, 1, 2, 4, 8, 10 h after the start of NO_2-fumigation, frozen in liquid nitrogen immediately, and stored at -80°C. Total RNA was extracted according to the procedure

of Chirgwin et al. (1979). Total RNA was loaded (10 μg per line) on 1% agarose gel containing 2 M formaldehyde, and electrophoresed. Trans to Hybond-N Plus nylon filter (Amersham, Buckinghamshire, England) and UV-crosslinking to immobilized RNA was done as described by Church and Gilbert (1984). Filters were hybridized to gene probes in 50% formamide, 1% SDS, 10% dextran sulfate, 0.05 M Na-Pi buffer, 5×standard saline citrate (SSC; 1×SSC=150 mM NaCl, 15 mM sodium citrate) and 100 μg ml^{-1} denatured salmon sperm DNA at 42°C for 12 to 24 h. ^{32}P-labeled gene probes were prepared by random primer method (T7 Quick Prime Kit; Pharmacia Inc., Piscataway, NJ, USA) using 3'UTR fragments of the GS1 or GS2 cDNA (see above), and 0.9 kb *Bam* HI-28S rRNA fragment from pED30 plasmid which containing *Arabidopsis thaliana* 28S rRNA cDNA, as templates. Filters were then washed three times in 2×SSC, 1% SDS at 42°C for 5 min and twice in 0.1×SSC, 0.1% SDS at 65°C for 15 min. Quantitative analysis of the hybridization signals derived from GS1 and GS2 transcripts, which were calibrated with the signals from 28S rRNA, was done using a BAS 3000 Bio-Imaging Analyzer (Fuji Film Co., Tokyo, Japan).

RESULTS

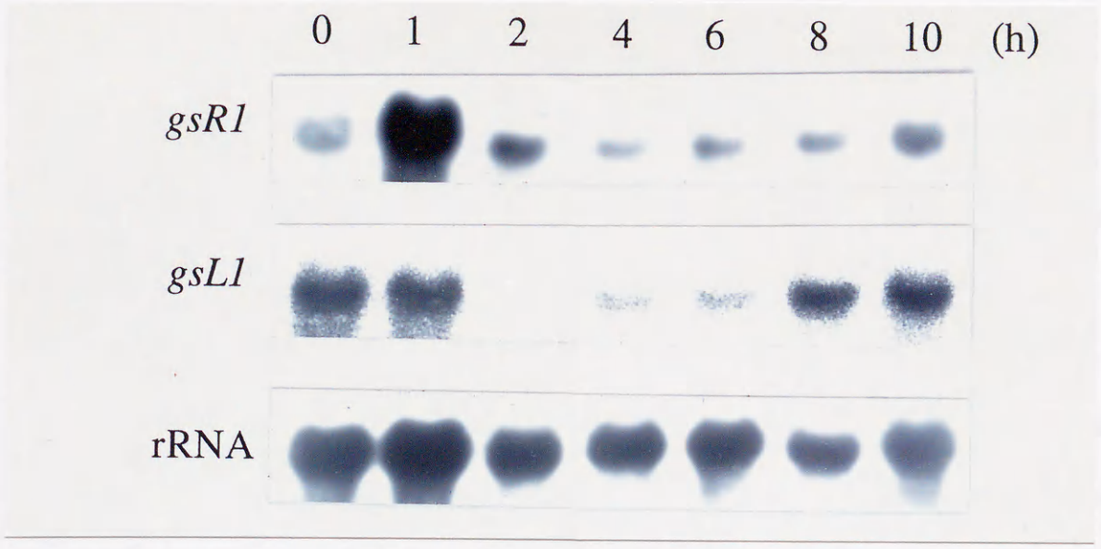
Fig. 1 shows changes of relative levels of *gsR1* (GS1) and *gsL1* (GS2) mRNAs in *Arabidopsis thaliana* leaves fumigated with 4 ppm NO_2 for up to 10 h. Their coding regions had approximately 70% identity while the identity of the UTR's was less than 40%. So, these UTR sequences were used as probes for Northern blot analysis. The GS1 mRNA level increased 2.5 times in response to fumigation with NO_2 1 h after the start of fumigation. Then it decreased to almost 60% of the peak level in another 1 h and reached more or less the initial level 10 h after the start. On the other hand, the GS2 mRNA level decreased drastically to 22% of the initial level

2 h after the start. It was still low another 4 h and increased to almost same level of the start for up to 10 h.

DISCUSSION

I found that the GS1 and GS2 mRNA levels changed in response to 4 ppm NO_2 fumigation by Northern blot analysis. However, the response was drastically different in each mRNA. The GS1 mRNA increased transiently 1 h after the start of fumigation and the GS2 mRNA decreased 2 h after the start, and they were back to the level of the start for up to 10 h. The *Arabidopsis thaliana* nitrate reductase (NR) and nitrite reductase (NiR) mRNAs also increase transiently, peaked 1 h after the start of fumigation, in response to 4 ppm NO_2 when plants had been previously starved for nitrate for 1 week (Morikawa et al. 1998b). The responses of these mRNAs to NO_2 are similar to that of GS1 mRNA rather than that of GS2 mRNA. These gene expressions may be regulated by the similar transcriptional signals.

The cytosolic GS1 isoform locates in the vascular system (Carvalho et al. 1992, Kamachi et al. 1992, Marsolier et al. 1995), and GS2 located only in the mesophyll cells of their leaves (Edwards et al. 1990). Their gene expressions are also different in plant organ, and are thought to be regulated by different signal factors, e.g., nitrate, nitrite, amino acids as like glutamine and glutamate, or light. When *Arabidopsis thaliana* plants were fumigated with 4 ppm NO_2 for 4 h, the 2.4- and 1.5-folds of the accumulation of nitrite and ammonium ions were detected in their leaves, respectively (data not shown). Such nitrogen compounds may activate and/or repress their gene expressions. With the aim of improving our understanding of GS gene regulation, several researches will be performed, e.g., investigating of transgenic plants and an immunochemical analysis of GS isozymes.



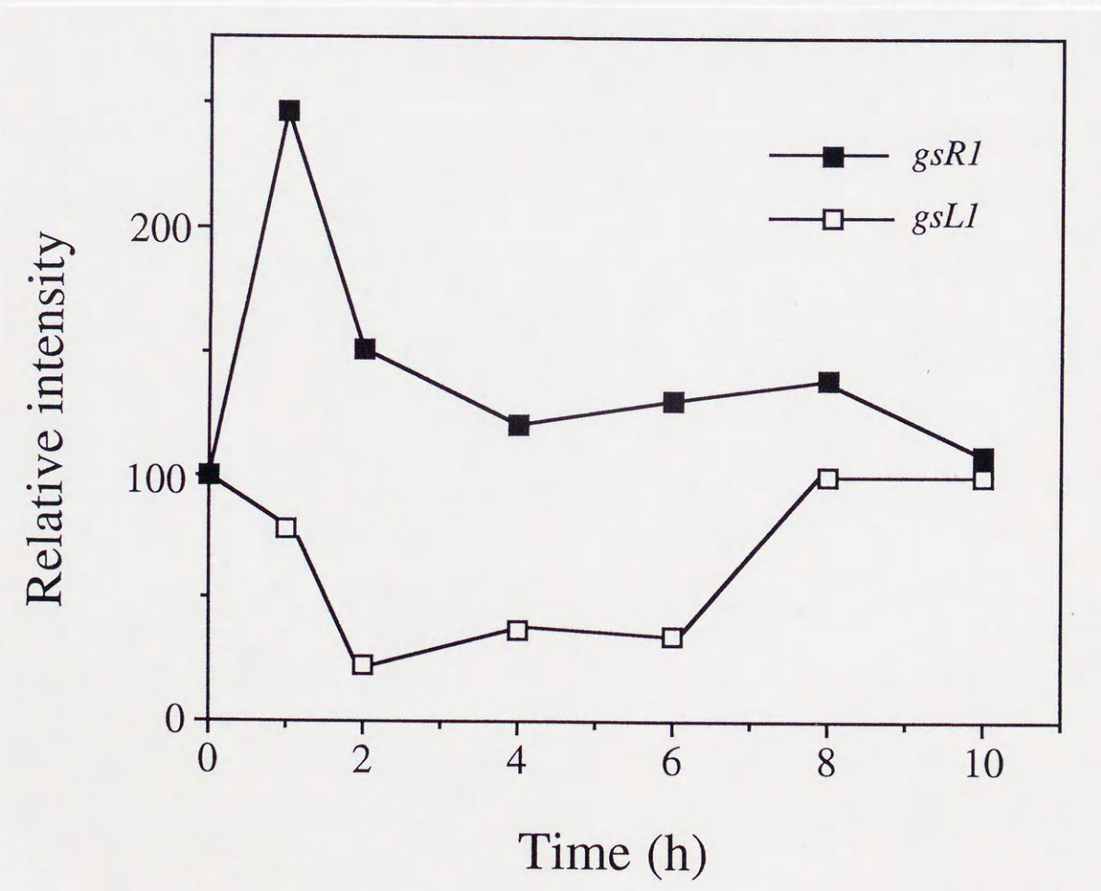


Fig. 1 Time course of the changes of GS1 and GS2 mRNA levels in response to 4 ppm nitrogen dioxide in *Arabidopsis thaliana* leaves. The GS mRNA levels in each lane was normalized against that of rRNA level.

CHAPTER III

in planta Assay for The Effect of Antisense GS cDNA Introduced into *Arabidopsis thaliana* Leaves by Particle Bombardment

GS is a key enzyme in nitrogen metabolism in various organisms (McNally and Hirel 1983), and in higher plants GS plays an essential role in the assimilation of ammonia generated from various reduction processes including nitrate reduction and photorespiration (Key et al. 1978, Miflin and Lea 1980, Wallsgrove et al. 1987, Edwards and Coruzzi 1989). Plants can uptake nitrogen dioxide (NO_2) through stomata in leaves and assimilate its nitrogen into amino acids and proteins through the GS /glutamate synthetase (GOGAT) pathway in plastids (Yoneyama et al. 1979, Wellburn 1990).

In this chapter, I show the new method of efficient delivery of transgenes into the leaves that were attached to an intact *Arabidopsis thaliana* plant by particle gun. Successful transient expression of *uid* A under the control of CaMV 35S promoter and NOS polyadenylation signal is described here. Using this method, an expression vector for an antisense GS cDNA was bombarded into the leaves attached to an intact *Arabidopsis thaliana* plant. Bombarded plants were subjected to standing in the atmosphere without added NO_2, or to fumigation with 4 ppm nitrogen dioxide (NO_2), and analyzed biochemically. I describe the results of enzyme activity analysis, Western blot analysis, and ion content analysis.

MATERIALS AND METHODS

Plant material

The growth conditions of *Arabidopsis thaliana* ecotype C24 were described in chapter II. Prior to further experiments for *in planta* assay, the plants were transferred to a fumigation chamber and kept in the chamber with no added NO_2 for 24 h under the conditions described in chapter II.

Bombardment

Plants in pots were taken out from the fumigation chamber, and a disk of 0.6% agarose gel was (or not) placed underneath the leaves of a target plant on the surface of the soil in a pot so that each of the leaves was solidly held onto the gel surface. Then the plant in a pot was placed in he bombardment box of the particle gun device (Rehbock model 260, Rehbock Co., Japan; Morikawa, et al., 1994), and bombarded with gold particles coated with or without plasmid DNA as reported previously (Morikawa et al., 1989; Iida et al., 1990) with slight modifications; 4 μg of plasmid DNA per mg of gold particles (1.1 μm, diameter, Tokuriki Honten Co., Tokyo, Japan) , 0.1 or 0.2 mg of DNA-coated gold particles per projectile, 6 cm of sample-to-stopper distance, and 375 m s^{-1} of initial velocity of the projectile. Two to six shots were given to each target plant. After being bombarded and the agarose gel plate being removed from the pots, bombarded plants were transferred back to the humidity-controlled chamber (70$\pm$4%). Some other plants were treated in the same way as for bombarded plants except that shots were given with projectiles without gold particles, after which these nonbombarded control plants also were transferred back to the chamber. These bombarded and nonbombarded plants were subjected to fumigation experiments.

Fumigation experiments

To the fumigation chamber, in which bombarded or nonbombarded plants had been placed, NO_2 gas at the concentration of at 4.0 ± 0.3 ppm NO_2 was applied, and plants were kept for up to 2 h in the condition described in chapter II. Standing treatments of bombarded or nonbombarded plants in the atmosphere were made exactly the same ways as fumigation treatment except that no NO_2 gas was added.

Cloning of GS cDNA and construction of plasmid DNA

Total RNA was isolated from leaves of 5-week-old plants and used for a template for the amplification of the first strand of GS cDNA using an RNA PCR Kit (Perkin Elmer Applied Biosystems Co., Ltd., Foster City, CA, U.S.A.). A pair of PCR primers (5'-AGTGATTCTATACCCGCAAGC-3' and 5'-CTCTCGTTTCCTTCACCGTAG-3'), which corresponds respectively to nucleotide bases from 452 to 472 and those from 1094 to 1114 in the sequence of a GS cDNA clone (*gsL1*) encoding GS2 polypeptide (Peterman and Goodman, 1991). Thirty-five cycles of amplification, each of which consisted of 1 min at 95°C for denaturation, 2 min at 65°C for annealing, and 1 min at 72°C for extension, were made. A 663-bp product thus obtained was cloned using pCR-Script SK(+) Cloning Kit (Stratagene, La Jolla, CA, U.S.A.). The nucleotide sequence of a clone (pAGSLF1) was identical to that of the corresponding region of GS2 cDNA (Peterman and Goodman, 1991). A 704-bp *Bam* HI/*Sac* I fragment from pAGSLF1 was inserted into the 3.8-kb deletion vector, in which the *uid*A was deleted from pBI221 by digestion with *Bam*HI and *Sac*I, and this leads to plasmid pSG. Note that the GS cDNA fragment was connected to cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthetase (NOS) polyadenylation signal in an antisense orientation.

Protein extraction and enzyme assay

All leaves were harvested from a bombarded target plant, and from them total soluble protein extracts were prepared at 4°C by homogenization of tissues in grinding buffer using a mortar and pestle. The grinding was prepared as 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 1.7% polyvinylpyrrolidone (pH 7.6). The extracts were clarified by centrifugation for 10 min at 18,000×*g*. Protein concentration of the extracts were determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard. GS enzyme activity in the extracts was determined according to Rhodes et al. (1975).

Western blot analysis

The proteins in the leaf extracts were analyzed by 10% SDS-polyacrylamide gel electrophoresis (PAGE) according to Laemmli (1970). Proteins were transferred electrically from SDS-PA gel to nitrocellulose filters following the procedure of Towbin et al. (1979). Following transfer, the filters were blocked overnight in TBST (20 mM Tris-HCl pH 7.6, 1 mM EDTA, 150 mM NaCl, 0.005% Tween 20) containing 2% skim milk (Difco Laboratories, Detroit, MI, U.S.A.). Afterwards, the blocking solution was substituted by TBST containing rabbit anti-lettuce GS1 antibody (a gift from Dr. Go Takeba, Kyoto Prefectural University) and incubated for 2 h. The blot was washed three times with TBST and incubated with [^{125}I]-goat anti-rabbit IgG for another 1 h. After being washed in TBST, the filters were subjected to autoradiography and quantitative analysis of the signal intensities was made by using BAS 3000 Bio-imaging analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Determination of the contents of NO_3^-, NO_2^- and NH_4^+ ions

Leaf tissues (50 mg fresh weight) were homogenized in 25 μl of 0.1% SDS and subsequently 200 μl chloroform using a mortar and pestle made of agate (Sanplatec Corp., Tokyo, Japan) for determination of NO_3^- and NO_2^- ion content. For determination of NH_4^+ ion content, leaf tissues were homogenized in 400 μl of ultrapure water using the teflon mortar and pestle. The homogenates were clarified by centrifugation twice at 18,000$\times g$ for 10 min, after which the respective ion contents were analyzed by using a capillary ion analyzer (Kawamura et al. 1996). The amount of total chlorophyll in the extracts was determined according to the method of Mackinney et al. (1941). The ion contents were expressed in μmoles or nmoles per mg of total chlorophyll of the sample.

RESULTS

GUS expression in intact leaves attached to Arabidopsis thaliana *plant*

I studied the efficient bombardment of transgenes into the leaves that were attached to an intact *Arabidopsis thaliana* plant. The leaves that were being held or not held onto the gel plate were bombarded with pBI221 (CaMV 35S pro-*uid*A gene-NOS ter) and then subjected to histochemical assay for GUS expression. The results are summarized in Table 1. Clearly, in the bombarded leaves holding onto the agarose gel plate, there was more blue spots of GUS-expressing cells than in the leaves not held to the plate. At most, a total of 515 spots on GUS expression cells per plant were observed for two shots of 0.2 mg gold particles per projectile each, in which more than 50 of the blue spots per leaf were detected. In the following experiments, bombardments were made to the attached leaves that were held to agarose plate.

Table 1 Transient expression of the GUS gene in the leaves attached to *Arabidopsis thaliana* plants

Experiment No.	Holding of leaves [a]	mg Au / projectile	No. of shot	No. of GUS-expression units / plant [b]
1	-	0.1	2	4
2	-	0.1	4	2
3	-	0.1	4	2
4	-	0.1	4	6
5	-	0.2	2	3
6	-	0.2	2	4
7	-	0.2	2	5
8	-	0.2	2	9
9	+	0.1	4	63
10	+	0.1	4	148
11	+	0.1	4	222
12	+	0.1	4	292
13	+	0.1	6	225
14	+	0.2	2	125
15	+	0.2	2	502
16	+	0.2	2	515
17	+	0.2	3	108
18	+	0.2	3	157
19	+	0.2	3	380

[a] The leaves, which were in the state of attachment to the plants, were held (+) or not (-) on a disk of agarose gel.

[b] The number of GUS-expression units (blue spots) in a plant.

Changes in GS activity

Fig. 1 shows changes in GS activity in the leaves of *Arabidopsis thaliana* plants as a function of time for standing in the atmosphere without added NO_2 (Fig. 1A) or for fumigation with 4 ppm NO_2 (Fig. 1B). Prior to "standing" or "fumigation" treatment, plants were not bombarded (thereafter they are designated as NB plants), or mock bombarded, i.e., bombarded with noncoated gold particles (MB plants), or bombarded with pSG-coated gold particles (SG plants). When NB and MB plants were stood in the atmosphere without added NO_2, no appreciable changes in the enzyme activity were observed. On the other hand, when these plants were subjected to fumigation with NO_2, gradual increases in GS activity in the leaves were observed. Thus, fumigation treatment of nonbombarded or mock bombarded *Arabidopsis thaliana* plants with NO_2 seems to induce an increase in GS activity in their leaves. When SG plants were stood in the atmosphere, GS activity in the leaves decreased gradually over 2 h. Thus, introduction of an antisense GS cDNA seem to cause a decrease in GS enzyme activity in *Arabidopsis thaliana* leaves. This decrease in GS activity in SG plants became more pronounced upon fumigation with NO_2. Reason(s) for this is not fully understood yet. It is likely that fumigation with NO_2 is harmful to SG plants (see below).

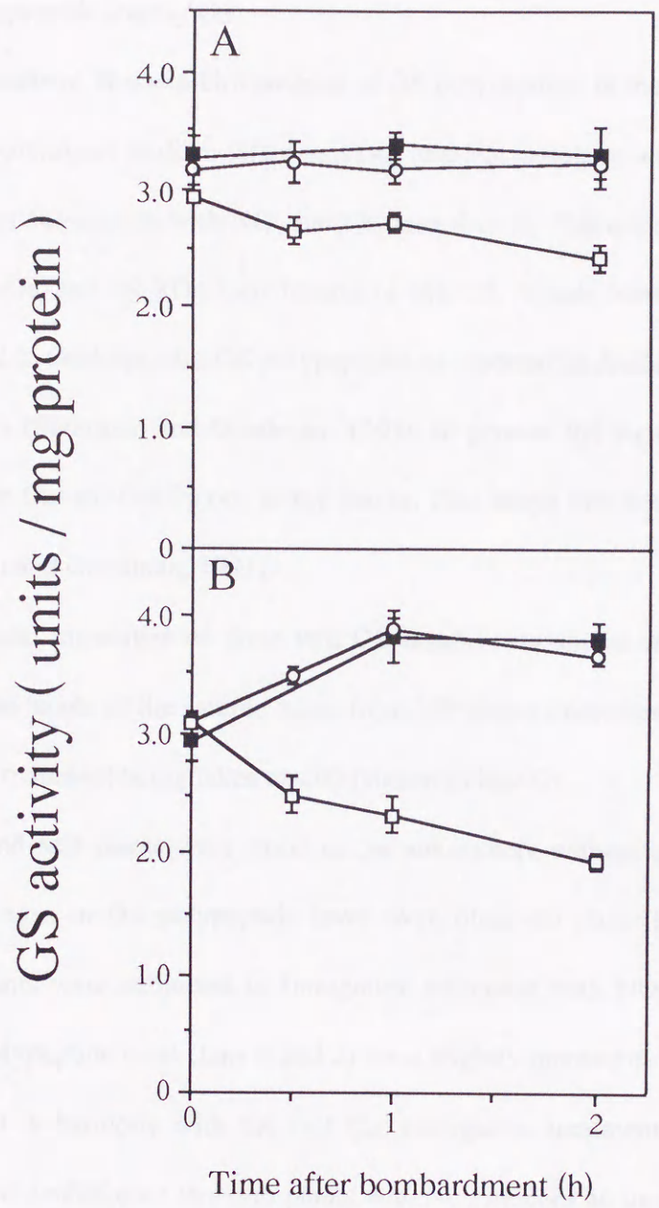


Fig. 1 Changes in GS activity in the leaves of *Arabidopsis thaliana* plants. Nonbombarded (■), mock bombarded (○), and pSG-bombarded (□) plants were subjected to standing treatment in the atmosphere without added NO_2 (A). NB, MB and SG plants were subjected to fumigation with 4 ppm NO_2 (B). One unit of GS activity is defined as 1 μmol of γ-glutamylhydroxamate formed per min. Each point represents the mean of results from 3 to 4 plants with SD indicated by vertical lines.

Changes in polypeptide level of GS

Fig. 2 shows results of Western blot analysis of GS polypeptides in the leaves of NB, MB and SG plants of *Arabidopsis thaliana* after standing in the atmosphere without added NO_2 for 2 h (lane 1 to 3) or fumigation with NO_2 for 2 h (lane 4 to 6). There were observed two bands around at 43 kDa and 39 kDa (see inserts in Fig. 2), which correspond respectively to chloroplast- and cytosol-specific GS polypeptides as reported in *Arabidopsis thaliana* by the previous authors (Peterman and Goodman, 1991). In general the signal of the 39-kDa band was weaker than that of 43-kDa one in the leaves. This keeps line with the previous authors' result (Peterman and Goodman, 1991).

Change in total intensities of these two GS bands were shown in Fig. 2B, where total intensity of these bands of the sample taken from NB plants immediately before standing-in-the-atmosphere treatment being taken as 100 (shown in lane 0).

When NB and MB plants were stood in the atmosphere without added NO_2 for 2 h, no appreciable changes in GS polypeptide level were observed (lane 1 and 2, respectively). When these plants were subjected to fumigation treatment with NO_2 for 2 h, the average values of GS polypeptide level (lane 4 and 5) were slightly increased over the control plants. The latter result is harmony with the fact that fumigation treatment of nonbombarded or mock bombarded *Arabidopsis thaliana* plants with NO_2 induces an increase in GS activity in their leaves as shown in Fig. 1. When SG plants were stood in the atmosphere for 2 h, GS polypeptide level in the leaves distinctly decreased (lane 3). Similar decrease in the level of GS polypeptides was observed when SG plants were fumigated with NO_2 (compare lane 3 with 6). These results keep line with the observed decreases in GS enzyme activity as described above (see Fig. 1).

Visible damages of leaves upon fumigation with NO_2

Fig. 3 shows *Arabidopsis thaliana* plants before (A) and after (B) bombardment of a plasmid pSG followed by fumigation with NO_2 for 2 h. Clearly, almost all of the leaves were more or less damaged in fumigated SG plants (compare A and B). These visible damages on the leaves became detectable approximately 30 min after the start of fumigation. On the other hand, as shown in Fig. 3C, no visible damages were detected on their leaves when pSG-bombarded plants were stood in the atmosphere without added NO_2. Also, MB plants did not show any visible damages on their leaves when they were stood in the atmosphere or fumigated with NO_2 (data not shown). It is thus conceivable that visible damages on the leaves observed when SG plants were fumigated with NO_2 are attributable to the decrease in the level of the activity of GS enzymes due to introduction of antisense GS cDNA. Decrease in GS enzyme level may result in the accumulation of ammonium ion in leaf cells, which is harmful to the cells. Thus, effects of bombardment of plasmid pSG and fumigation with NO_2 on the ion contents in the leaves were analyzed.

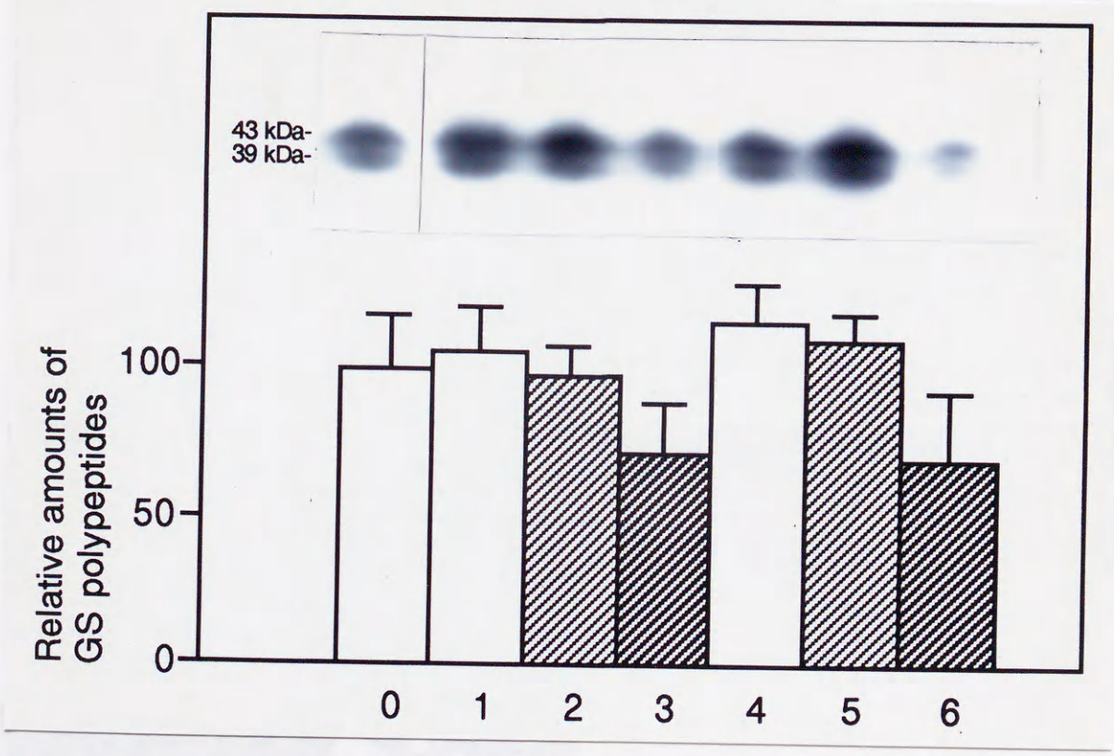


Fig. 2 Western blot analysis of GS polypeptides in the leaves of *Arabidopsis thaliana* plants. Nonbombarded, mock bombarded and pSG-bombarded plants were subjected to standing treatment in the atmosphere without added NO_2 for 2 h (lanes 1, 2 and 3, respectively). NB, MB and SG plants were subjected to fumigation with 4 ppm NO_2 for 2 h (lanes 4, 5 and 6, respectively). Lane 0 shows the result of the sample taken from nonbombarded plants immediately before standing-in-the-atmosphere treatment. The size of the two bands is shown at the left of the inserts. The relative amounts of GS polypeptides estimated from total intensities of these two bands (the mean of 3 experiments with SD indicated by vertical lines) are shown in the bottom where the result of lane 0 being taken as 100.

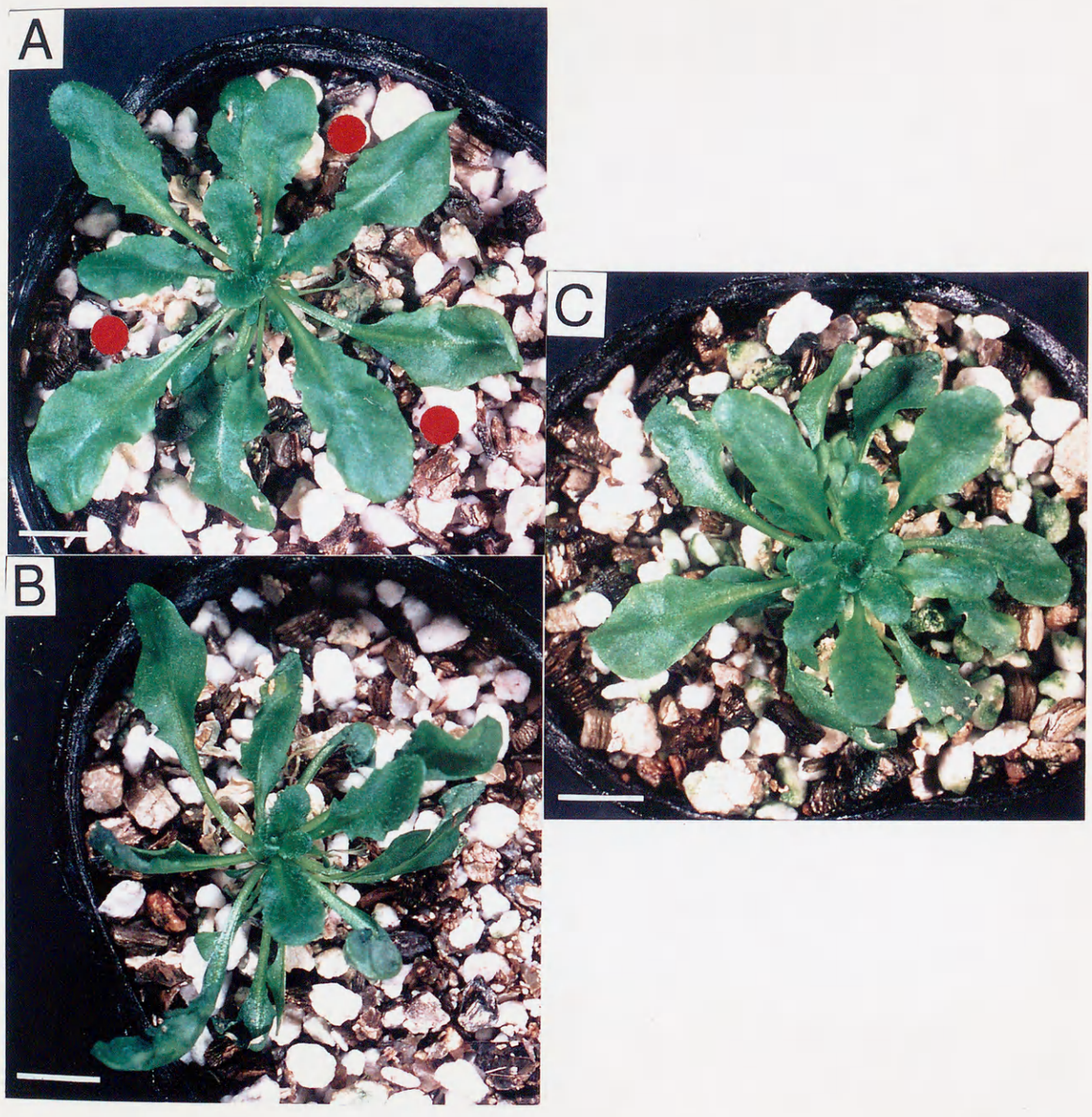


Fig. 3 Photographs of *Arabidopsis thaliana* plants before (A) and after (B and C) bombardment of an antisense GS cDNA. A plant was bombarded three times with plasmid pSG at the positions indicated by red dots in (A). This plant was then subjected to fumigation treatment with NO_2 for 2 h (B). Another plant was bombarded in the same way and stood in the atmosphere without added NO_2 for 2 h (C). Bar represents 1 cm.

Analysis of the contents of NO_3^-, NO_2^- and NH_4^+ ions

Table 2 shows the contents of NO_3^-, NO_2^- and NH_4^+ ions in the leaves of NB, MB and SG plants of Arabidopsis thaliana that were not fumigated (i.e., stood in the atmosphere without added NO_2) or fumigated with NO_2 for 2 h. The values of NO_3^- content of these three types of *Arabidopsis thaliana* plants with or without fumigation treatment were coincided within experimental errors, indicating that the NO_3^- content in the leaves is rather stable and not affected by fumigation with NO_2 and/or by bombardment of antisense GS cDNA. Similar results were obtained with NH_4^+ content except that the content of this ion significantly increased, not by fumigation with NO_2 but by bombardment of plasmid pSG; 4.6 ± 0.3, 6.1 ± 0.5 and 6.0 ± 0.6 μmol mg^{-1} total chlorophyll (average of 3 experiments $\pm$ SD), respectively, for nonfumigated NB plants, nonfumigated SG plants and fumigated SG plants.

Fumigation treatment of NB plants tended to increase NO_2^- content in the leaves; the average value of the content increased ca. 1.7 times upon fumigation. Similar result was obtained with MB plants. However, due to relatively large experimental errors in these cases whether or not NO_2^- content in the leaves of *Arabidopsis thaliana* plants is increased in response to fumigation treatment can not be concluded. NO_2^- content in the leaves of nonfumigated SG plants did not differ from that of nonfumigated NB or MB plants. Thus, bombardment of plasmid pSG itself did not seem to affect NO_2^- content, which is in contrast to the case of NH_4^+ content (see above). On the other hand, fumigation of SG plants markedly increased NO_2^- content in the leaves; 5.7 ± 2.7 and 22.8 ± 4.5 (nmol mg^{-1} total chlorophyll) respectively before and after fumigation. This four-fold increase in NO_2^- content in the leaves of SG plants could have caused visible damages of their leaves in response to fumigation with NO_2.

Table 2 Effects of bombardment of pSG and fumigation with NO_2 on ammonia, nitrite and nitrate content in *Arabidopsis thaliana* leaves

Bombardment	Ammonia [a] (μmol mg^{-1} total chlorophyll)		Nitrite (nmol mg^{-1} total chlorophyll)		Nitrate (μmol mg^{-1} total chlorophyll)	
	Nonfumigated[b]	Fumigated[c]	Nonfumigated	Fumigated	Nonfumigated	Fumigated
None	4.6±0.3	3.9±0.5	7.2±2.4	12.3±3.5	154.0±29.7	117.0±29.5
Noncoated gold particles	4.4±1.1	4.2±0.5	5.4±1.6	8.6±0.8	123.6± 5.2	116.5± 5.1
pSG-coated gold particles	6.1±0.5	6.0±0.6	5.7±2.7	22.8±4.5	132.2±41.9	111.4± 7.1

[a] Each value is the mean of results of 3 plants with SD.
[b] Stood in the atmosphere for 2 h.
[c] Fumigated with 4 ppm NO_2 for 2 h.

DISCUSSION

The present results showed that bombardment of an antisense GS cDNA fragment into the leaves of *Arabidopsis thaliana* attached to intact plants by particle bombardment induced important changes in biochemical characteristics of the plant leaves; distinct decreases in both enzyme activity and polypeptide level of GS (see Figs. 1 and 2), and an increase in NH_4^+ content (see Table 2). This result can best be interpreted as an indication that transient expression of the introduced antisense GS cDNA induced inhibition of a part of de novo synthesis of GS polypeptides, which resulted in a decrease in the level of GS activity and increased in the NH_4^+ content. Because the cDNA fragment of GS used here has 100 identity with leaf-specific GS2 cDNA and 70 to 73% identity with root-specific GS1 cDNA (Peterman and Goodman 1991), it is likely that the synthesis of both types GS polypeptides are inhibited by the introduction of the antisense GS cDNA. Fumigation treatment of pSG-bombarded plants (SG plants) with NO_2 strengthened the decreases in both GS enzyme activity and polypeptide level in their leaves. Molecular mechanism of this "additive effect" of the fumigation treatment is not known. Fumigation treatment of SG plants induced a four-fold increase in the content of nitrite ions in the leaves. Thus, this increase in the level of the toxic ion could have caused damages in the cells of the leaves as shown in Fig. 3, and these damages are likely causes for accelerated decreases in the enzyme activity and polypeptide level.

The present result provides evidence that introduction of transgenes into intact tissues attached onto intact plants by a particle gun, and subsequent biochemical and molecular biological analyses of the bombarded tissues *(in planta* assay) will be a rapid and useful tool for the study of the function of transgenes in plant cells.

CHAPTER IV

Stable Transformations of *Arabidopsis thaliana* Introduced with the GS1 and GS2 cDNAs in a Sense or Antisense Orientation

The different GS isozymes assimilate ammonia produced by different physiological processes (Edwards et al. 1990, Lam et al. 1995) and probably have different physiological requirements for optimal activity (Cullimore and Bennett 1988, Temple et al. 1996). Chloroplastic GS2 has been proposed to serve two functions in leaves. One is the assimilation of ammonia reduced from nitrate or nitrite in the plastid (Fentem et al. 1983), and the second is in the assimilation of photorespiratory ammonia (Wallsgrove et al. 1987, Lam et al. 1995). On the other hands, cytosolic GS1 is thought to assimilates ammonia released by breakdown of nitrogenous resources during germination in roots and germination seeds (Walker and Coruzzi 1989, Peterman and Goodman 1991, Watanabe et al. 1994), and in leaves and stems it is postulated that it functions to generate glutamine for nitrogen transport (Edwards et al. 1990, Kamachi et al. 1991). However little is known about it.

With aim of gaining a better understanding of functions of GS isozymes, I attempted to alter GS levels in *Arabidopsis thaliana* by introduction of plasmids that contain a full length or fragment of GS1 or GS2 cDNA in sense or antisense orientation under the control of CaMV 35S promoter and NOS polyadenylation signal. In this report, I will briefly discuss the results investigated about their transformations.

MATERIALS AND METHODS

Plant materials

Seedlings of *Arabidopsis thaliana* ecotype C24 were grown, and root sections were prepared as described previously (Sawasaki et al. 1994). Briefly, four- to five-five old seedlings that had been aseptically grown on germination medium (GM) containing MS salts and vitamins (Murashige and Skoog 1962), 3% sucrose and 0.6% agarose under 16 h light / 8 h dark at 22˚C were harvested, and roots were excised and cut into sections (0.5-1 cm long). Approximately 200 root sections were spread in circle (35 mm diameter) on a filter paper on callus-inducing medium (CIM) containing MS salts and vitamins, 2% sucrose, 0.5 mg l^{-1} 2,4-dichlorphenoxyacetic acid, 0.05 mg l^{-1} kinetin and 0.3% gellan gum (pH 5.6). After being cultured for three days under the conditions described above, they were subjected to particle bombardment.

Plasmid DNA

The *Xba* I-*Eco* RI fragments containing approximately 1335-bp of *gsR1* (GS1) cDNA and 1545-bp of *gsL1* (GS2) cDNA, respectively, were inserted into the 3.8-kb deletion vector in sense orientation, in which the *uid*A was deleted from pBI221 by digestion with *Xba* I and *Eco* RI. These lead to plasmids pAGS1 and pAGS2, respectively. On the other hand, The *Sac* I-*Xho* I fragment containing 153-bp of 3'UTR for GS1 cDNA and *Sac* I -*Xba* I fragment containing 164-bp of 3'UTR for GS2 cDNA, respectively (see chapter II), were inserted into the 3.8-kb deletion vector which the *uid*A was deleted from pBI221, in the antisense orientation. These lead to plasmids pASG1 and pASG2, respectively. Note that the GS cDNA fragments were connected to cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthetase

(NOS) polyadenylation signal (Fig. 1).

Bombardment

The pneumatic particle gun device and the methods for gene delivery to root sections using this device were essentially as reported previously (Takahashi and Morikawa 1996) with slight modifications. The plasmid DNA, pAGS1, pAGS2, pASG1, or pASG2 was separately mixed with pCH (CaMV 35S promoter-*hpt*-NOS terminator) (Goto et al. 1993) in a molar ratio of 1:1 and co-precipitated in ethanol. Bombardment conditions were as follows: 4 μg of plasmid DNA per mg of gold particles (1.1 μm, diameter, Tokuriki Honten Co., Tokyo, Japan), 0.2 mg of DNA-coated gold particles per projectile, 10 cm of sample-to-stopper distance, and 375 m s^{-1} of initial velocity of the projectile. Two shots were given to each target tissue sample.

Selection of hygromycin-resistant calluses

After being bombarded, the root sections on the filter papers were cultured on CIM for 3 days (Takahashi and Morikawa 1996). The root sections were then divided into small clumps, and they were transferred onto shoot-inducing medium (SIM) containing MS salts and vitamins, 3% sucrose, 5 mg l^{-1} N^6-(2-isopentenyl)adenine, 0.3 mg l^{-1} IAA, and 0.3% gellan gum (pH 5.6), and supplemented with 20 mg l^{-1} hygromycin. They were cultured, by being transferred fortnightly onto a fresh SIM, under the same conditions as described above. Two to four weeks after bombardment, green spots consisting of the hygromycin-resistant callus became clearly visible on callus clumps.

Formation of transgenic plants from hygromycin-resistant calluses

Approximately five weeks after bombardment, hygromycin-resistant shoots were formed from the green spots of hygromycin-resistant calluses. The shoots were allowed to grow on the same medium (hygromycin-containing SIM) for another three to fifteen weeks to confirm their hygromycin resistance, after which they were transferred onto hygromycin-free shoot-elongation medium (SEM) containing MS salts and vitamins, 1% sucrose and 0.3% gellan gum in a cylindrical plastic container (ca. 7 cm diameter and 11 cm tall; Agripot, Kirin Co., Tokyo, Japan) to allow their rapid elongation for two to five weeks. Each of the elongated putative transgenic shoots was then detached from the callus clump and transplanted onto hygromycin-free rooting medium (RM) containing MS salts and vitamins, 1% sucrose, 1 mg l^{-1} IAA, and 0.3% gellan gum in the container for root formation. About two weeks after the transplantation, the shoots were rooted. The plants were then transferred to a rock wool irrigated with a half-strength solution of MS salts and allowed to set seeds by self-pollination.

PCR analysis

Genomic DNAs were extracted from *Arabidopsis thaliana* leaves (Saghai-Maroof et al. 1984). Each PCR amplification of the trans-gene fragment containing partially CaMV 35S promoter and each GS cDNA was performed with the genomic DNA as a template, respectively. A sense primer sequence were 5'-GACGCACAATCCCACTATCC-3' which locate in nucleotide positions from 795 to 814 for CaMV 35S promoter. Antisense primer sequence used for the each amplification of cDNA carrying the plasmid pAGS1, pAGS2, pASG1 or pASG2 were 5'GACTTATCCTAAGACATTGC-3', 5'-TTCAAATCCTCCTTCCTCTC-3', 5'-CCGGATCCGATGATCATCAGATC-3', and 5'-

CCCGAATTCGAATCTGATGAACACACGTG-3', which correspond respectively to nucleotide 1217 to 1236, 1024 to 1043, 1133 to 1147, or 1390 to 1409 in the sequence of GS1 or GS2 cDNA. Thirty-five cycles of amplification, each of which consisted of 1 min at 95°C for denaturation, 2 min at 60°C for annealing, and 1 min at 72°C for extension, were made.

Assays of enzyme activity and Western blot

Arabidopsis thaliana plants were grown in vermiculite and perlite for 5 weeks described in chapter II. The methods for the extraction of total proteins from the leaves, the protein quantification, and the assay of GS enzyme activity were described in chapter III. The procedures of SDS-PAGE and Western blot analysis for the detection of GS polypeptide signals were basically followed as described in chapter III, but the GS signals on the filter were detected by reaction with a peroxides labeled goat anti-rabbit IgG (VECTOR Laboratories Inc., Burlingame, CA, U.S.A.) and subsequently ECL (Amersham Life Science, Buckinghamshire, England) after the reaction with rabbit anti-lettuce GS1 antibody. Quantitative analysis of the signal intensities was made by Quantity One Ver. 2.7.

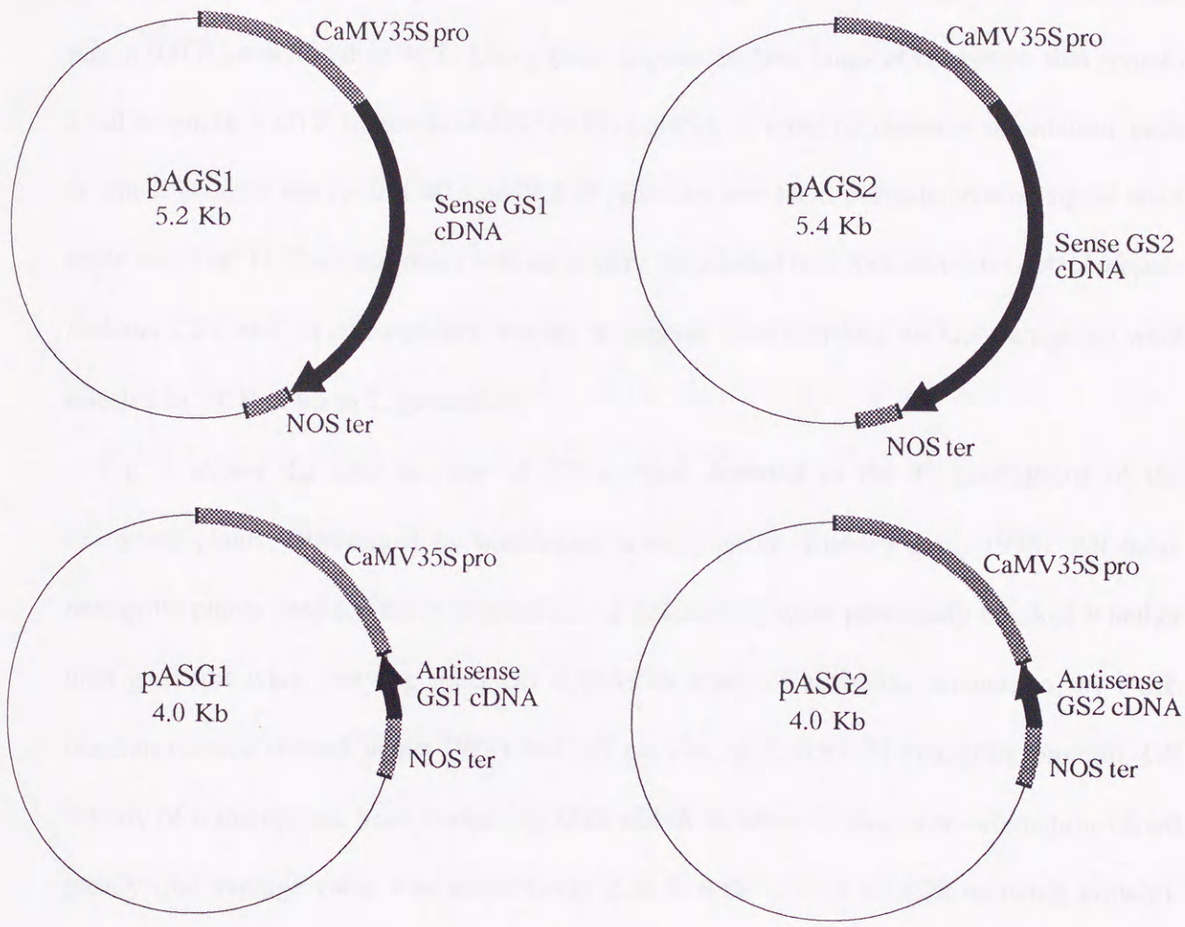


Fig. 1 Schematic maps of pAGS1, pAGS2, pASG1, and pASG2. Constracts introduced are pAGS1 containing full length (1335 bp) of GS1 cDNA in sense orientation, pAGS2 containing full length (1545 bp) of GS2 cDNA in sense orientation, pASG1 containing 153-bp of 3'UTR for GS1 cDNA in antisense orientation, and pASG2 containing 164-bp of 3'UTR for GS2 cDNA in antisense orientation. Each of cDNA fragments was under the control of CaMV 35S promoter (CaMV 35S pro) and nopaline synthetase polyadenylation signal (NOS ter).

RESULTS AND DISCUSSION

We have cloned cDNAs for *gsR1* (GS1) or *gsL1* (GS2) of *Arabidopsis thaliana* cotype C24. Their coding regions had approximately 70% identity while the identity of untranslated region (UTR) was less than 40%. Using these sequences, four kinds of constructs that contain a full length or 3'UTR fragment of GS1 or GS2 cDNA in sense or antisense orientation, each of which is under the control of CaMV 35S promoter and NOS polyadenylation signal were made (see Fig. 1). Each construct was separately introduced into root sections of *Arabidopsis thaliana* C24, and, in consequence, twenty transgenic lines carrying the GS transgenes were selected by PCR in up to T_2 generation.

Fig. 2 shows the total activity of GS enzyme detected in the T_2 generations of the transgenic plants, determined by transferase activity assay (Rhodes et al. 1975). All these transgenic plants used for the determination of GS activity were previously checked whether their genomes were carrying trans GS cDNAs in sense or antisense orientation, by PCR. Nontransformed control plants (WT) had GS activity of 2.35 ± 0.31 (units/mg protein). GS activity of transformant lines containing GS2 cDNA in sense or antisense orientation varied greatly (the average value was respectively 2.28 to 4.88 or 0.91 to 4.28 units/mg protein). The lines 2A09, 2S10 and 2S11 had more than 2-fold higher GS activity than that of the control (4.28 ± 0.29, 4.88 ± 1.37 and 4.70 ± 0.75 units/mg protein, respectively). On the other hand, those lines containing GS1 cDNA in sense or antisense orientation showed much lower GS activity (the average value was respectively 0.70 to 1.01 or 1.18 to 2.92 units/mg protein), except a line (line 2A09). Co-suppression can be a cause for the suppressed GS activity in the transformants with GS1 cDNA in sense orientation.

Fig. 3 shows the signals of GS polypeptides in the T_2 generations of transgenic plants and

a control plant (WT) determined by Western blot analysis. There were observed two bands around at 43 kDa and 39 kDa (see inserts in Fig. 3), which correspond respectively to chloroplast GS2 and cytosolic GS1 polypeptides as reported in *Arabidopsis thaliana* by the previous authors (Peterman and Goodman, 1991). Lines 2S10 and 2S11 containing GS2 cDNA in sense orientation had higher level of GS2 polypeptides than the control (180 and 146% of the control, respectively), and also line 2S10 had more GS1 (123% of the control). Line 1A12 containing GS1 cDNA in antisense orientation had lesser GS1 (6% of a control plant) and the other line containing GS1 cDNA in antisense orientation had a new band below the GS1 signal. It may be formed the digestion of GS polypeptides. On the other hand, lines 1S01 and 1S24 had less polypeptides in both GS1 and GS2, regardless of containing GS1 cDNA in sense orientation. It agrees well with the results of GS activity in those lines. Also, it is likely that the synthesis of both types of GS polypeptides are slightly inhibited in lines 2A03 and 2A17 containing GS2 cDNA in antisense orientation.

Some transgenic plants enriched or reduced GS have been previously obtained (Temple et al. 1994, Kozaki and Takeba 1996, Temple and Sengupta-Gopalan, 1997), however, only a few molecular and biochemical analysis have been available so far as I know. I also successfully obtained some transgenic plants which were overexpressing or down-regulating GS isozymes, whereas I have not studied physiological and biochemical analyses in those transgenic lines yet. I think surely those transgenic lines would exhibit some physiological changes or different responses to the nitrogen-signals compared with the wild type plants, in consideration of the results for a potent inhibitor of GS (MSX) (see chapter V) and the effects of antisense GS cDNA in *in planta* assay (see chapter III). I currently am working on this, and results will be published elsewhere.

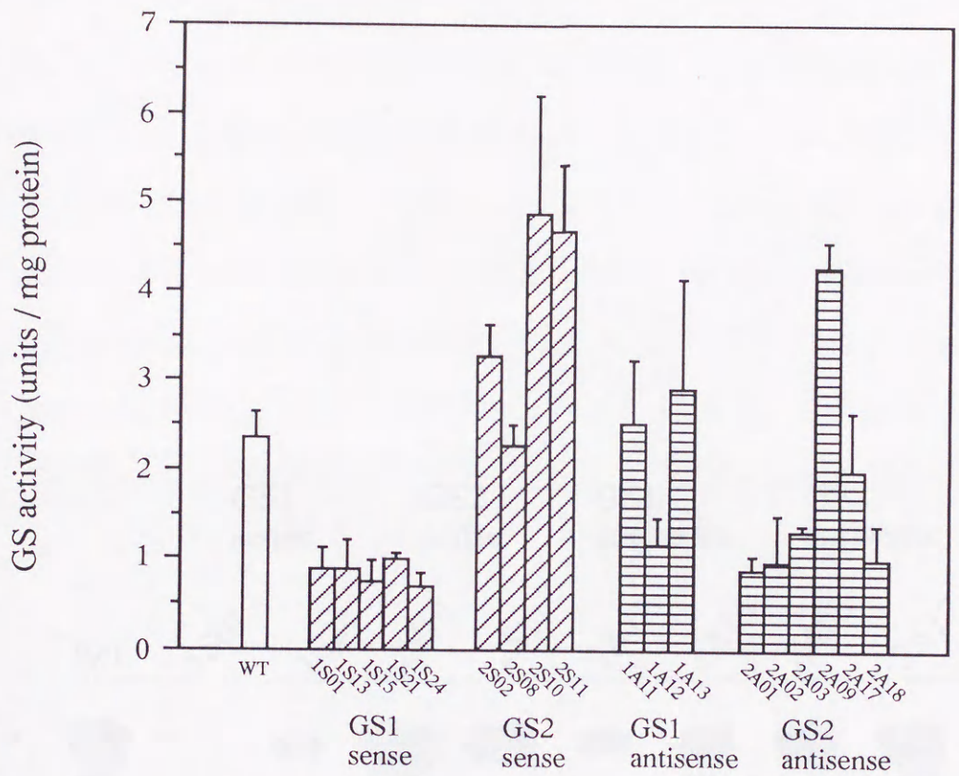


Fig. 2 GS enzyme activity in leaves of various transgenic lines of *Arabidopsis thaliana*. Leaves from five-weeks old transformed or untransformed (WT) *Arabidopsis thaliana* plants were harvested and total GS enzyme activity was determined by transferase activity assay after the protein extractions. One unit of GS activity is defined as 1 μmol of γ-glutamylhydroxamate formed per min. Values are means with SD of results for 2 to 4 samples.

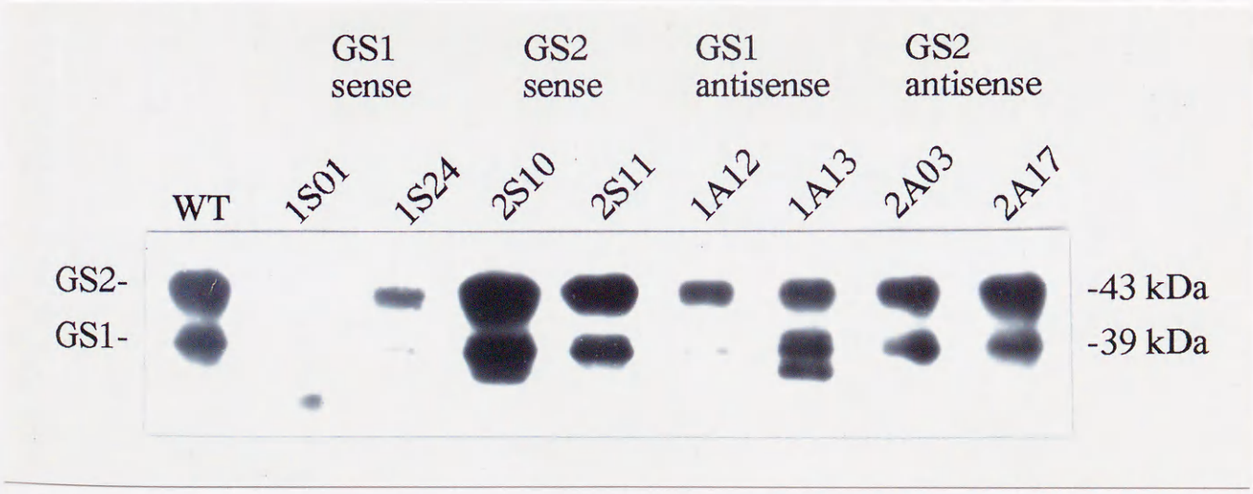


Fig. 3 Western blot analysis of GS polypeptides in various transgenic lines of *Arabidopsis thaliana*. Total soluble proteins of *Arabidopsis* wild type (WT) and each transgenic line were separated by 10% SDS-PAGE, blotted to nitrocellulose, and identified GS polypeptides by Western blot analysis. The molecular weight (kDa) of GS polypeptides is indicated on the right.

CHAPTER V

Metabolic fate of nitrogen dioxide nitrogen in plant leaves

Nitrogen dioxide (NO_2) is a major atmospheric pollutant, and plants act as a sink for it (Rogers et al. 1979, Yoneyama and Sasakawa 1979, Yoneyama et al. 1979, Okano et al. 1988, Morikawa et al. 1998a, Morikawa et al. 1998b). In aqueous solution (e.g., in the apoplastic water around leaf cells) NO_2 rapidly produces nitrate and nitrite ions which then are converted to organic compounds such as amino acids or proteins (Yoneyama and Sasakawa 1979, Kaji et al. 1980, Lee and Schwartz 1981, Wellburn 1990, Nussbaum et al. 1993) through the nitrate assimilation pathway. Formation of ^{15}N-labeled nitrate, nitrite, and amino acids in plants fumigated with ^{15}N-labeled NO_2 has been reported (Yoneyama and Sasakawa 1979, Kaji et al. 1980), and since then it has been assumed that NO_2 nitrogen is metabolized similarly to the nitrate in plant cells. Whether a stoichiometric relationship exists between the total nitrogen (N) derived from NO_2 and the sum of reduced and nitrate/nitrite N has not, however, been studied. I therefore used ^{15}N-labeled NO_2 ($^{15}NO_2$) and potassium nitrate ($K^{15}NO_3$) to clarify whether the total ^{15}N taken up in the leaves of *Arabidopsis thaliana* plants is equal to the amount of reduced ^{15}N plus nitrate/nitrite ^{15}N. I found that the metabolic fate of NO_2 nitrogen in the leaves differs from that of nitrate nitrogen.

MATERIALS AND METHODS

Plant material

The growth conditions of *Arabidopsis thaliana* ecotype C24 were described in chapter II. Seedlings of 5- to 6-week-old plants were used for the experiments.

Fumigation with $^{15}NO_2$ before and after treatment with MSX

The *Arabidopsis thaliana* seedlings in the plastic pots were placed in a fumigation chamber and treated with $^{15}NO_2$ (51.6 atom%) at concentrations of 0.1 to 4.0 ppm for up to 4 h under the conditions described in chapter III. Relative variation in the NO_2 concentrations among the experiments was less than 10%. The pots were covered with polyethylene bags to avoid dissolution of NO_2 into the soil layer. Alternatively, before fumigation with NO_2, 1 mM L-methionine sulfoximine (MSX, Sigma Chemical Co., St. Louis, MO, U.S.A.) solution was applied to the plants. They were kept for 24 h in the fumigation chamber under the conditions described, except that no NO_2 was added, after which they were fumigated with NO_2 as described above.

Treatment with $K^{15}NO_3$

Arabidopsis thaliana seedlings were treated with 1 mM $K^{15}NO_3$ (50.0 atom% ^{15}N), 20 or 50 mM $K^{15}NO_3$ (10.0 atom% ^{15}N) and kept in the fumigation chamber with no added NO_2 for up to 8 h under the conditions described above.

Preparation of leaf samples

Leaves were harvested from *Arabidopsis thaliana* plants treated with $^{15}NO_2$ or $K^{15}NO_3$ were rinsed with distilled water. Unless otherwise stated, the treated leaves were dried at 80°C for 12 to 24 h, then the dried leaves of 6 to 10 plants (50 to 150 mg DW) were combined and ground into powder with a mortar and pestle, and stored in a desiccator until used.

Determination of NO_2-derived (or nitrate-derived) nitrogen in the total, reduced, ammonium, and nitrate/nitrite N fractions

Total N fraction

Approximately 1 mg samples of the powdered leaves were weighed in containers made of tin for use in an elemental analyzer (EA/NA; Fisons Instrument, Milano, Italy). The ^{15}N (atom% excess) content was determined with a mass spectrometer (Delta C; Finnigan MAT, Bremen, Germany) connected directly to the elemental analyzer (EA/NA; Fisons Instrument, Milano, Italy). The amount of NO_2-derived (or nitrate-derived) nitrogen, here defined as the total nitrogen (total N) expressed in nanograms of nitrogen per milligram of dry leaves [ng N (mg DW)$^{-1}$], was estimated from these values.

Reduced N fraction

The reduced nitrogen (reduced N) fractions of the dried leaf samples were prepared by the Kjeldahl method, and the total amount of reduced N was determined by titration as described elsewhere (Morikawa et al. 1998a). To quantify the ^{15}N content in the distillates by mass spectrometry, the ammonia in the distillates was concentrated by the Conway diffusion method (Conway and Byrne 1933). Two milliliters of distillate was added to 2 ml of 5% boric acid buffer (pH 10) in the outer chamber of a Conway diffusion unit whose inner chamber

contained 55 μl of 0.5 N H_2SO_4. After the lids were secured, the units were kept at 25°C for 24 h. The distillate in the outer chamber then was replaced with fresh distillate, and the unit kept for another 24 h at 25°C. This procedure was repeated up to three times depending on the concentration of ammonia in the distillate. Fifty microliter portions of the solution were transferred from the Conway unit to the tin containers of the EA-MS analyzer, and the ^{15}N (atom% excess) content in each solution was quantified. Using the ^{15}N value and total amount of reduced N determined by titration, we estimated the quantity of NO_2-derived (or nitrate-derived) reduced N in each sample, expressed by [ng N (mg DW)$^{-1}$].

Ammonium N fraction

A mortar and pestle made of agate (Kawamura et al. 1996) was used to homogenize leaf powder (30 to 50 mg DW) in pure water from a Milli-Q system (7MQ2167, Nihon Millipore Kogyo K.K., Yonezawa, Japan) in order to prepare the ammonium nitrogen (ammonium N) fraction of the dried leaf samples. The homogenate was centrifuged twice at 18,000×*g* for 10 min. Total ammonia in the supernatant (sup) was determined by capillary electrophoresis (Kawamura et al. 1996). The ammonia in the sup was concentrated using Conway diffusion units as described above, and the ^{15}N (atom% excess) content of each solution was determined by mass spectrometry. Using these two values, we estimated the quantity of NO_2-derived (or nitrate-derived) ammonium N in each sample, expressed by [ng N (mg DW)$^{-1}$].

Nitrate/nitrite N fraction

Leaf powder (20 to 30 mg DW) in 0.1% SDS was homogenized with the agate mortar and pestle to prepare the dried-leaf fraction containing both nitrate and nitrite (nitrate/nitrite N fraction). The homogenate was centrifuged twice at 18,000 $\times g$ for 10 min. The total nitrate and nitrite in the sup of each homogenate was determined by capillary electrophoresis (Kawamura et al. 1996).

For quantification of the ^{15}N content in the fraction, the nitrate and nitrite in the homogenate were separated by ion exchange chromatography, 2 ml of the sup being applied to a column (0.8×1.3 cm) of Dowex 50W hydrogen form (Fentem 1983), and 5 ml of pure water added. The total eluates (7 ml) were combined and brought to 20 ml with pure water, to which 1 ml of 10 N NaOH and 0.8 g of Devarda alloy (100 mesh) had been added to reduce nitrate and nitrite to ammonia (Gatley and Shea 1991). The reaction mixture was left for 24 h at room temperature, after which the ammonia in it was recovered by distillation, as done with the Kjeldahl digests, then concentrated in Conway diffusion units as described above. The ^{15}N (atom% excess) content of each solution was determined by mass spectrometry. Using this value and the total amount of nitrate and nitrite determined by capillary electrophoresis (see above), we estimated the quantity of NO_2-derived (or nitrate-derived) nitrate/nitrite N in each sample, expressed by [ng N (mg DW)$^{-1}$].

Fractionation of leaf homogenate

Leaf powder samples (150 mg DW) prepared from *Arabidopsis thaliana* plants fumigated with 4 ppm $^{15}NO_2$ for 4 h were homogenized in 0.1% SDS. The homogenate then was centrifuged at 18,000×g for 10 min. The contents of NO_2-derived total and reduced N in the

sup and pellet fractions were determined by mass spectrometry as described above.

Preparation of symplast and apoplast solutions

Leaves were harvested from plants fumigated with 4 ppm $^{15}NO_2$ for 4 h, and symplast and apoplast solutions prepared by the method of Sakurai & Kuraishi (1988). A vacuum pump was used to infiltrate leaves from 7 plants (approximately 800 mg FW) with distilled water for 2 min. The leaves were cut into segments (approximately 0.5 cm long) and placed on a stainless-steel mesh in the barrel of a 2.5 ml plastic syringe. The whole was placed on an Eppendorf tube (1.5 ml) in a centrifuge tube and centrifuged at 8,000×*g* for 15 min to collect the apoplast solution (AP solution). To prepare the symplast solution (SY solution), segments were frozen in liquid nitrogen immediately after centrifugation, then thawed and recentrifuged at 8,000×*g* for 15 min to separate the cell sap (SY solution) and residue. The contents of NO_2-derived total and reduced N in the symplast and apoplast solutions and residue were determined by mass spectrometry.

Ion-exchange chromatography

Leaf powder samples (150 mg DW) prepared from *Arabidopsis thaliana* plants fumigated with 4 ppm $^{15}NO_2$ for 4 h were homogenized in pure water, after which the sup was obtained by centrifugation at 18,000×*g* for 10 min. Two milliliters of the sup was applied to a column (0.8×1.3 cm) of Dowex 50W hydrogen form, and 5 ml of pure water added. Combining the water eluates gave the non-cationic fraction. Next, 7 ml of 4 N HCl was applied to the column, and the resulting eluate gave the cationic fraction. The contents of NO_2-derived total and reduced N in the cationic and non-cationic fractions then were determined by mass

spectrometry.

Gel-permeation of chromatography

Leaf powder samples (150 mg DW) prepared from *Arabidopsis thaliana* plants fumigated with 4 ppm NO_2 for 4 h were homogenized in 3 mM Tris-HCl buffer containing 0.15 M NaCl (pH 7.0). The homogenate was centrifuged twice at 18,000×*g* for 10 min,. The sup was passed through a Superdex 75 HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden) and separated using a fast protein liquid chromatography (FPLC) system (Pharmacia Biotech, Uppsala, Sweden). The elution buffer was 3 mM Tris-HCl that contained 0.15 M NaCl (pH 7.0), and the flow rate 0.4 ml min^{-1}. Eluate fractions of 3.2 ml were collected. The NO_2-derived total N and reduced N contents of each fraction respectively were determined by mass spectrometry using 50 μl and 2.6 ml of the eluate.

RESULTS

Recovery rate of nitrogen

Table 1 shows the recovery rate of nitrogen from standard compounds before and after the various chemical and physical treatments. Direct analysis of their crystals or solutions gave almost 100% recovery. Kjeldahl digestion followed by distillation also gave almost 100% recovery. The ammonia concentration obtained by the Conway diffusion method, repeated three times, gave a recovery rate of 74.4±17.3%. Separation of nitrate by ion exchange in a Dowex 50W column followed by the reduction of nitrate to ammonia with Devarda alloy, and a single concentration of ammonia by the Conway diffusion method gave a high recovery rate (85.8±1.2%). It should be noted that the recovery rate of ^{15}N in the ammonia N fraction

and that of the nitrate/nitrite N fraction (see Materials and Methods) were estimated to be 100% because the recovery of ammonia, nitrate or nitrite determined by capillary electrophoresis was very close to 100% (Kawamura et al. 1996). Also, note that the values for ammonium nitrogen found by EA-MS, capillary electrophoresis, and titration coincided within the experimental errors.

Uptake rate of NO_2 and nitrate in Arabidopsis thaliana *plants*

Fig. 1 shows the uptake rate for 4 h in the light of the total N derived from $^{15}NO_2$ or $K^{15}NO_3$ in the leaves of *Arabidopsis thaliana* plants fumigated with 0.1 to 4 ppm $^{15}NO_2$ or fed 1 to 50 mM $K^{15}NO_3$ at their roots. The results agree well with those reported previously (Rogers and Campbell 1979, Ramge et al. 1993). Note that treatments with 0.1 ppm $^{15}NO_2$ and 1 mM $K^{15}NO_3$ gave very similar contents for the total N derived from $^{15}NO_2$ and nitrate in the cells. This also was the case with 4 ppm $^{15}NO_2$ and 50 mM $K^{15}NO_3$.

Upon fumigation with 0.1 or 4 ppm $^{15}NO_2$ for 4 h or treatment with 1 or 50 mM $K^{15}NO_3$ for 4 h, the nitrate ion level in the leaves of *Arabidopsis thaliana* plants, determined by capillary electrophoresis (Kawamura et al. 1996), was 10 to 15 μg N (mg DW)$^{-1}$. The nitrite ion level, however increased with fumigation, e.g., 2.4-fold with 4 ppm $^{15}NO_2$ for 4 h, but not with the nitrate-treatments.

Table 1 Recovery of nitrogen before and after various chemical and physical treatments of standard compounds

Treatment	Sample	Recovery (%)
None [a]	Crystals [b]	100.0 ± 0.5
	KNO_3 or $(NH_4)_2SO_4$ solution [c]	97.3 ± 1.9
Kjeldahl digestion and distillation [d]	Glutamine or $(NH_4)_2SO_4$ solution	98.6 ± 4.3
Conway diffusion method (three times) [e]	$(NH_4)_2SO_4$ solution	74.4 ± 17.3
Ion exchange, reduction, and Conway diffusion method [f]	KNO_3 solution	85.8 ± 1.2

[a] Direct measurement in an EA-MS analyzer.

[b] Crystals (0.5 to 1 mg) of acetanilide, cyclohexanone-2,4-dinitrophenylhydrazone, or atropine were weighed in the tin container and placed in the sample port of the EA-MS analyzer to quantify the nitrogen content of the sample.

[c] Fifty microliters of 5,000 ppm KNO_3 or $(NH_4)_2SO_4$ solution was placed in the tin container, and dried. Then the nitrogen content was determined in an EA-MS analyzer.

[d] One milliliter of 10,000 ppm glutamine or $(NH_4)_2SO_4$ solution was placed in 20 ml of Kjeldahl digestion solution. The mixture was digested, and the ammonia in it distilled. The ammonia in the distillates was determined by titration.

[e] Ammonia, in 6 ml of 1,000 ppm $(NH_4)_2SO_4$ solution, was concentrated in the Conway diffusion unit. The nitrogen content was determined in an EA-MS analyzer.

[f] Two milliliters of 5,000 ppm KNO_3 solution was applied to a Dowex 50W column. The nitrate fraction was separated and collected, then reduced by Devarda alloy to ammonia. The ammonia was concentrated in the Conway diffusion unit. The nitrogen content was determined in an EA-MS analyzer. See text for details.

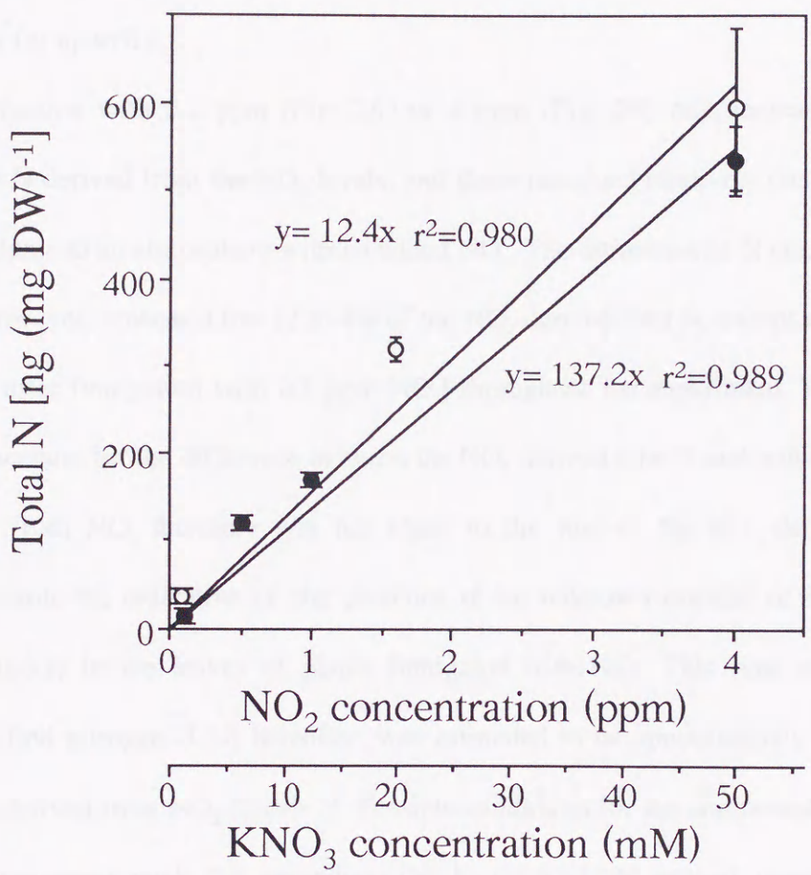


Fig. 1 The amount of total N taken up by leaves of *Arabidopsis thaliana* plants fumigated with $^{15}NO_2$ or fed $K^{15}NO_3$. Plants were fumigated with 0.1, 0.5, 1, or 4 ppm $^{15}NO_2$ for 4 h (●), or fed 1, 20, or 50 mM $K^{15}NO_3$ for 4 h (○). Leaves were harvested, and their total N contents determined. Values are means with SD of results for three samples.

Metabolic fate of NO_2-N and nitrate-N

Fig. 2 shows the total, reduced, and nitrate/nitrite N derived from NO_2 or nitrate in the leaves of *Arabidopsis thaliana* seedlings fumigated with $^{15}NO_2$ (0.1 or 4 ppm) for up to 4 h then transferred to an atmosphere without added NO_2 for another 8 h and in roots fed $K^{15}NO_3$ (1 or 50 mM) for up to 8 h.

Fumigation with 0.1 ppm (Fig. 2A) or 4 ppm (Fig. 2B) NO_2 increased the total and reduced N derived from the NO_2 levels, and these remained relatively constant after transfer of the plants to an atmosphere without added NO_2. The nitrate/nitrite N derived from the NO_2 level, however, remained low [2 to 4% of the NO_2-derived total N, except at 1 h (13%) and 2 h (7%) after fumigation with 0.1 ppm NO_2] throughout the experiment. The value was too low to account for the difference between the NO_2-derived total N and reduced N. The total N derived from NO_2 therefore was not equal to the sum of the NO_2-derived reduced and nitrate/nitrite N, indicative of the presence of an unknown organic or inorganic nitrogen compound(s) in the leaves of plants fumigated with NO_2. This type of nitrogen, called unidentified nitrogen (UN) hereafter, was estimated to be approximately 20 to 30% of the total N derived from NO_2 (Table 2). Possible candidates for the compound(s) containing UN are organic compounds that are indigestible by the Kjeldahl method, inorganic ones that are difficult to reduce to ammonia by the Devarda alloy, or both.

In the case of treatment with 1 mM (Fig. 2C) or 50 mM (Fig. 2D) $K^{15}NO_3$, the levels of the total, reduced, and nitrate/nitrite N derived from nitrate increased almost linearly with the period of treatment, and the nitrate-derived total N coincided, within the experimental errors, with the sum of the nitrate-derived reduced N and nitrate/nitrite N, much less or no UN being present in the leaves of plants fed $K^{15}NO_3$ (Table 2). It should be noted that in the case of

treatment with $K^{15}NO_3$, substantial amounts of nitrate-derived total N (40 to 60%) were accounted for by the nitrate/nitrite N, in marked contrast to the finding for NO_2-derived nitrogen. These results are clear evidence that the metabolic fate of NO_2 nitrogen differs from that of nitrate nitrogen in leaves of *Arabidopsis thaliana* plants.

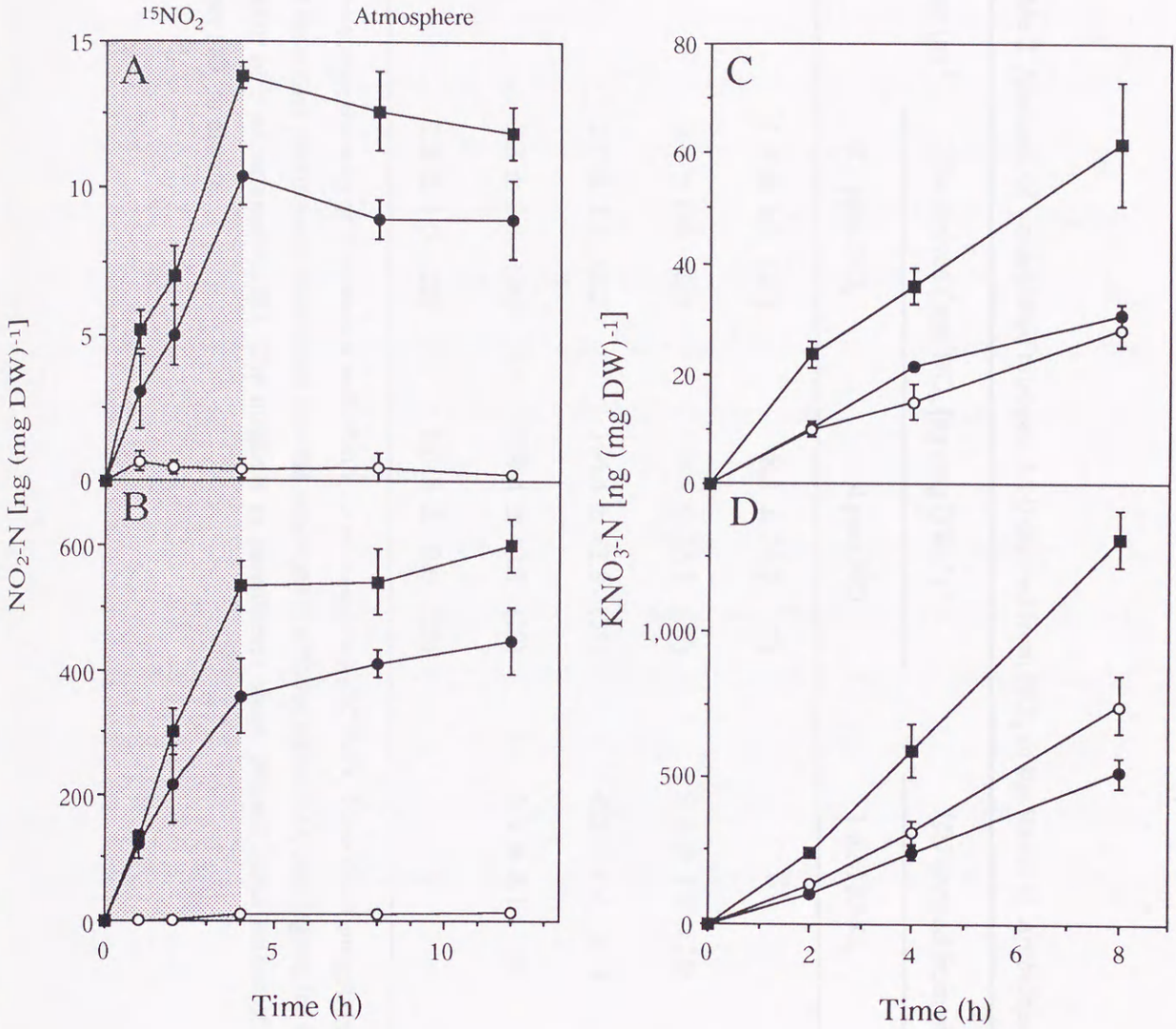


Fig. 2 Amounts of total, reduced, and nitrate/nitrite N in leaves of *Arabidopsis thaliana* plants fumigated with $^{15}NO_2$ or fed $K^{15}NO_3$. Plants were fumigated with 0.1 (A) or 4 (B) ppm $^{15}NO_2$ for up to 4 h then transferred to an atmosphere without added NO_2 and kept there for 4 to 8 h. Alternatively, plants were fed 1 (C) or 50 (D) mM $K^{15}NO_3$ for up to 8 h. The leaves were harvested at various times, and their total (■), reduced (●) and nitrate/nitrite (○) N determined. Values are means with SD of results for three samples.

Table 2 Amounts of unidentified nitrogen (UN) derived from NO_2 in the leaves of *Arabidopsis thaliana* plants

Time (h) [a]	UN derived from NO_2 [ng (mg DW)$^{-1}$] [b]		UN derived from nitrate [ng (mg DW)$^{-1}$] [b]	
	0.1 ppm NO_2	4 ppm NO_2	1 mM KNO_3	50 mM KNO_3
1	1.4 ± 1.6 (27)	8.8 ± 34.3 (7)	-	-
2	1.5 ± 0.8 (22)	79.5 ± 26.1 (27)	3.5 ± 1.8 (15)	-0.6 ± 11.2 (0)
4	3.0 ± 1.1 (22)	164.4 ± 42.8 (31)	-0.5 ± 0.4 (-2)	40.1 ± 38.0 (7)
8	3.3 ± 2.3 (26)	119.4 ± 42.3 (22)	2.5 ± 8.1 (4)	49.2 ± 96.0 (4)
12	2.8 ± 1.0 (23)	139.5 ± 8.6 (23)	-	-

[a] Time after the start of fumigation with $^{15}NO_2$ or treatment with $K^{15}NO_3$. Note that fumigation was terminated at 4 h, and thereafter plants were transferred into the atmosphere without added NO_2 (see legend for Fig. 2).

[b] Mean of 3 experiments ± SD. The numbers in parentheses show percent mean values of the total N derived from either NO_2 or nitrate.

Effects of MSX treatment

To clarify whether the UN in fumigated *Arabidopsis thaliana* plant leaves is formed before or after the reduction of NO_2 to ammonia, we studied the effect of MSX, a potent inhibitor of glutamine synthetase (GS) which assimilates ammonia into organic matter (Lea 1991). Fig. 3 shows the NO_2-derived total, reduced, and ammonium N, and UN contents of *Arabidopsis thaliana* plants treated (A) or not treated (B) with MSX before fumigation with 4 ppm $^{15}NO_2$.

MSX treatment decreased the amount of the NO_2-derived total N to almost half that of the control. In the MSX-treated plants, the NO_2-derived ammonia content was almost the same as that of the NO_2-derived reduced N, whereas the ammonia level in the control was less than 14% of the total N derived from NO_2. These results are consistent with the activity of GS enzyme detected in the leaves of MSX-treated plants, determined by transferase activity assay (Rhodes et al. 1975), being 3 to 11% that of the control 2 and 8 h after the start of fumigation. UN contents in MSX-treated plants ranged from 29 to 38% of the total N derived from NO_2, values close to or slightly higher than the values for the control (without MSX). This suggests that the compound(s) bearing the UN is formed before the reduction of NO_2 nitrogen to ammonia.

Stability of UN

Arabidopsis thaliana plants were fumigated with 4 ppm $^{15}NO_2$ for 4 h then kept for 1 week in pots. The NO_2-derived total and reduced N, and UN contents were analyzed daily from day 0 to 7. Both the NO_2-derived total N and UN in leaves were relatively constant even 1 week after fumigation, which suggests that the UN fraction is scarcely metabolized during this period (data not shown).

Fractionation of the leaf homogenate

To further clarify the characteristics of the UN, leaf homogenates prepared from *Arabidopsis thaliana* plants fumigated with 4 ppm $^{15}NO_2$ for 4 h were fractionated into sup and pellet fractions, and their NO_2-derived nitrogen contents analyzed (Fig. 4A). The total N derived from NO_2 was distributed in the sup and pellet fractions in the ratio of 7 to 3. The NO_2-derived total N and reduced N contents were almost the same in the pellet but differed in the sup fraction. This suggests that the UN is present in the sup but not the pellet fraction. On the average, 18 percentage points of UN were estimated to be recovered in the sup fraction, indicative that the most of the UN is located in this fraction.

When the sup fraction was separated in a Dowex 50W column, about 90% of the NO_2-derived total N was recovered in the cationic fraction, and the rest in the non-cationic fraction (Fig. 4B). About 11 percentage points of the UN were estimated to be recovered in the cationic fraction, but only 1 percentage point of UN in the non-cationic fraction. This suggests that the most of the UN-bearing compound(s) is cationic.

Fumigated plant leaves were fractionated into apoplast (AP) and symplast (SY) solutions and a residue. The NO_2-derived total N and reduced N contents of each of these fractions were analyzed (Fig. 4C). Glucose-6-P dehydrogenase activity, measured by the method of Brulfert *et al.* (1973), indicated that contamination by SY solution in the AP solution was less than 0.1%. About 69% of the NO_2-derived total N in the leaves was recovered in the SY solution, the ratio of total N to reduced N being approximately 6 : 4. Almost all the NO_2-derived nitrate/nitrite N was recovered in the SY solution. NO_2-derived total N and reduced N contents were almost the same in the AP solution and the residue, but not in the SY solution (see Fig.

4C), suggesting that the UN is present only in the SY solution. The SY solution was estimated to contain 22 percentage points of UN.

The sup fraction of the leaf homogenate prepared from *Arabidopsis thaliana* plants fumigated with 4 ppm $^{15}NO_2$ for 4 h was separated by gel-permeation chromatography (GPC). Each of the GPC fractions was analyzed for NO_2-derived total N and reduced N (Fig. 4D). Approximately 8, 66, and 7% of the NO_2-derived total N of the sup fraction were recovered, respectively, in fractions 4, 5 and 6. Most of the UN appeared to be contained in fraction 5, with some in fraction 6. When nitrate (mol wt = 62), phenylalanine (165), and cystine (240) were co-chromatographed, fraction 5 contained 91% of the nitrate, 100% of the phenylalanine, and no cystine. The compound(s) bearing the UN appears to have a low molecular weight similar to the weights of nitrate and phenylalanine. Fraction 5 was estimated to contain 18 percentage points of the UN. Preliminary analysis of the fraction 5 by GC/MS detected ^{15}N-bearing N-nitrosodiphenylamine (mol wt = 198), which accounted for about 10% of the UN.

To determine whether the UN-bearing compound(s) is water- or lipid-soluble, 150 mg of the dry matter of the fumigated leaves was homogenized in 3 ml sodium phosphate buffer (pH 7.0), after which 700 μl of the sup fraction was mixed with an equal amount of ether. Analysis of the excess ^{15}N in the ether and water layers showed that almost 100% of the total N derived from NO_2 was recovered in the water layer, therefore the UN-bearing compound(s) is hydrophilic.

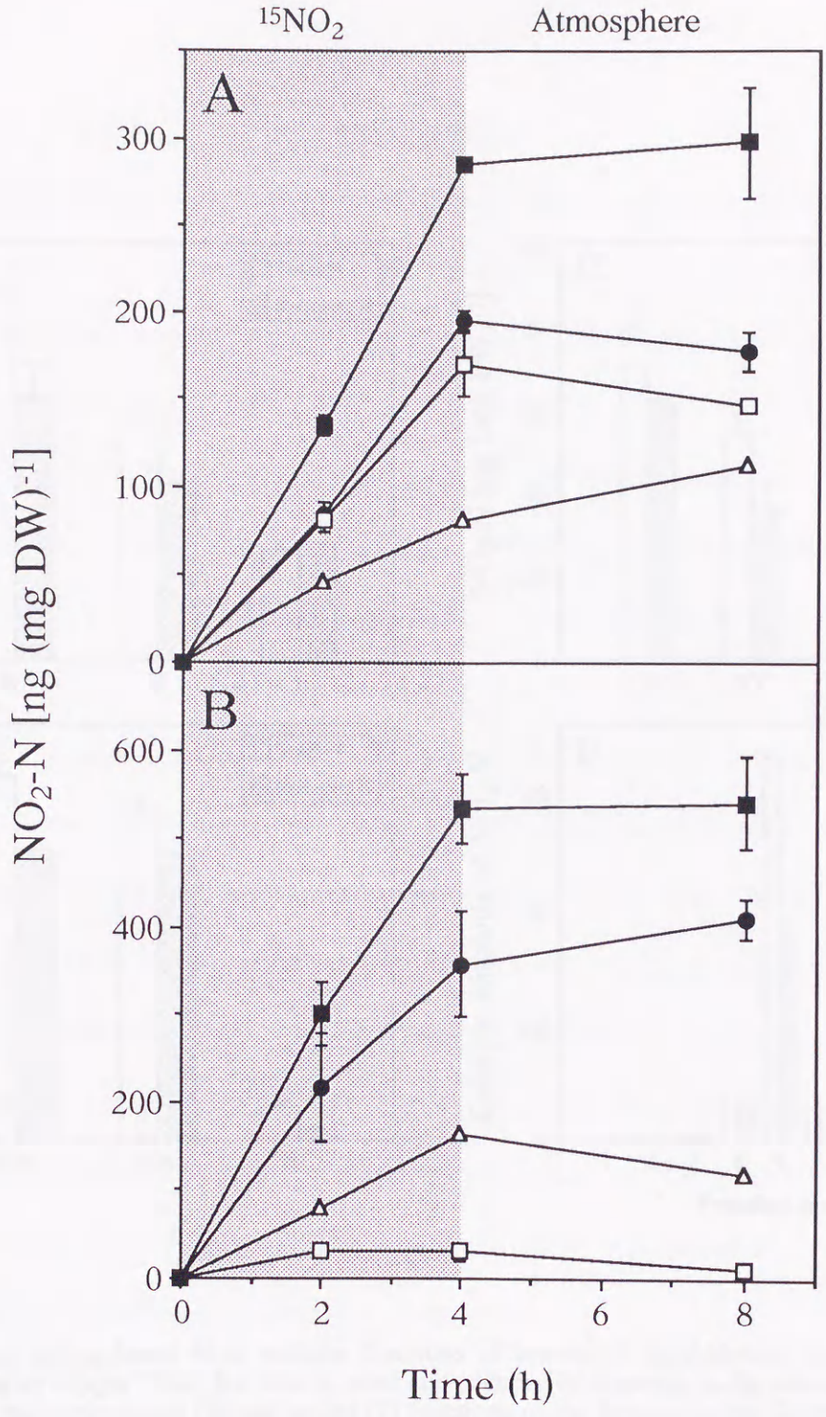


Fig. 3 Effects of MSX treatment on the total, reduced, ammonium, and unidentified N contents of *Arabidopsis thaliana* leaves. Plants were treated (A) or not treated (B) with 1 mM MSX for 24 h followed by fumigation with 4 ppm $^{15}NO_2$ for 4 h, then transferred to an atmosphere without added $^{15}NO_2$ for another 4 h. Leaves were harvested at various times, and total (■), reduced (●), ammonium (□), and unidentified (△) N contents determined. Values are means with SD of results for three samples..

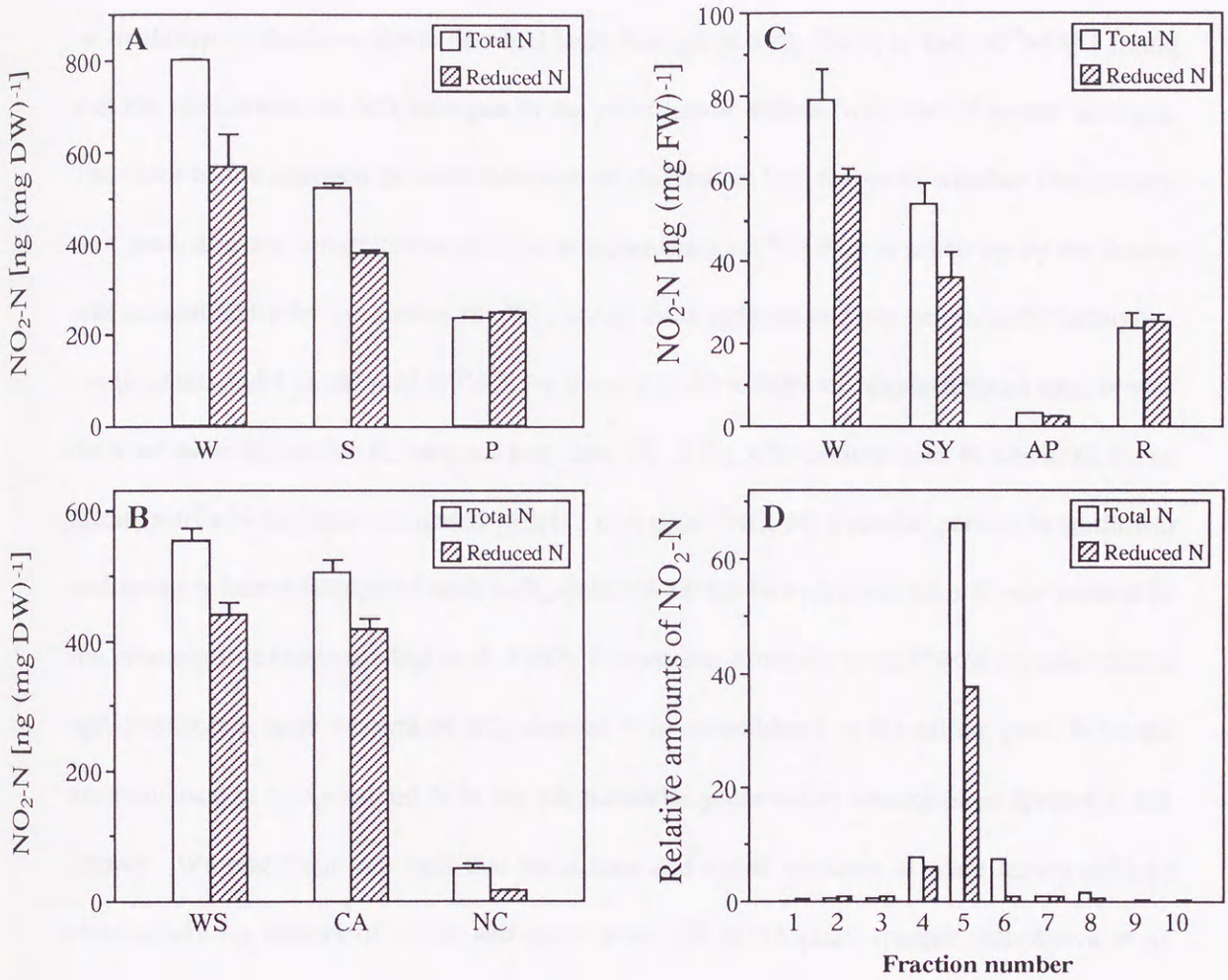


Fig. 4 Total and reduced N in various fractions of leaves of *Arabidopsis thaliana* plants fumigated with 4 ppm $^{15}NO_2$ for 4 h. A, total and reduced N contents in the whole leaf matter (W) and in the supernatant (S) and pellet (P) fractions of the homogenates. B, the contents of total and reduced N in cationic (CA) and non-cationic (NC) fractions separated by ion-exchange chromatography in Dowex 50W as well as the values in the entire supernatant before separation (WS). C, the total and reduced N contents in the symplast (SY) and apoplast (AP) solutions and residue (R), and their values in the whole leaf matter (W). D, contents of the total and reduced N fractions of the supernatant separated by gel-permeation chromatography in Superdex 75 HR 10/30. In A to C, the columns show the means of results for three samples, and the bars the SD.

DISCUSSION

In my analysis of the excess ^{15}N in the total, reduced, and nitrate/nitrite N fractions of leaves of *Arabidopsis thaliana* plants that had been fumigated with $^{15}NO_2$ or fed $K^{15}NO_3$, I found that the metabolism of NO_2 nitrogen in the plant leaves differs from that of nitrate nitrogen. The fates of the nitrogen of these compounds differed in two respects; whether the nitrogen was pooled in the nitrate/nitrite pool or whether the total ^{15}N excess taken up by the leaves was accounted for by the sum of the ^{15}N excess of the reduced and nitrate/nitrite N fractions.

In plants fed 1 or 50 mM $K^{15}NO_3$ for up to 8 h, 40 to 60% of nitrate-derived total N was detected as nitrate/nitrite N, whereas less than 4% of the NO_2-derived total N was detected as nitrate/nitrite N in plants fumigated with 0.1 or 4 ppm $^{15}NO_2$ for a similar period. In sunflower and spinach leaves fumigated with NO_2, only 1% of the NO_2-derived total N was present in the nitrate/nitrite fraction (Kaji et al. 1980). In contrast, Ammann et al. (1995) reported that in spruce shoots a large amount of NO_2-derived N is accumulated in the nitrate pool. Why the accumulation of NO_2-derived N in the nitrate/nitrite pools varies among plant species is not known. We elsewhere reported that the nitrate and nitrite contents of plant leaves differed respectively by factors of 5,000 and more than 100 in 11 plant species (Morikawa et al. 1998b). Differences in the pool sizes of nitrate and nitrite in plant species may affect NO_2 nitrogen accumulation in the nitrate/nitrite pools.

Twenty to 30% of the total N derived from the NO_2 taken up by the leaves of *Arabidopsis thaliana* plants fumigated with 0.1 or 4 ppm NO_2 could not be accounted for by reduced N or nitrate/nitrite N. This difference was designated UN (unidentified nitrogen). An MSX study indicated that this UN is formed before the conversion of NO_2 to organic matter. In the fractionation of leaf samples from fumigated plants, the UN was recovered in the sup of the

symplast fraction of the leaf cells, suggesting that this compound(s) is soluble in the leaf cell cytoplasm. Ion exchange chromatography indicated that the compound(s) bearing the UN is cationic, and gel permeation chromatography that it has a molecular weight similar to the weights of nitrate and phenylalanine.

Compounds bearing oxidized forms of nitrogen, organic nitro, nitroso, and azo compounds are known not to be converted to ammonia under the Kjeldahl digestion conditions I used (Christian 1986, Day and Underwood 1986). In fact, no ammonia was detected after the digestion of acetaldehyde oxime, nitrotyrosine, KNO_3, $NaNO_2$, and N_2O under those conditions. This suggests that organic nitro, nitroso, and azo, or the inorganic nitrous oxide used are candidates for the UN-bearing compound(s). Sodium thiosulfate as an additional oxidizing agent with copper sulfate as the catalyst in the Kjeldahl digestion mixture effectively reduces oxidized inorganic nitrogen to ammonia (McKenzie and Wallace 1953, Pace et al. 1982). Kjeldahl digestion of leaf samples under the conditions I used reduced the UN content by about 10 percentage points. Therefore, the UN-bearing compound(s) can not be reduced to ammonia even under those digestion conditions.

Nitrogen dioxide has an unpaired electron which make it a strong one electron oxidant (Beckman 1996), and it efficiently initiates free radical oxidation of unsaturated lipids, thiols, and proteins (Pryor and Lightsey 1981, Pryor et al. 1982, Prütz et al. 1985, Ramge et al. 1993) to form nitroderivatives such as nitrotyrosine. Nitro and nitroso compounds are difficult to digest by the Kjeldahl method (see above). The UN-bearing compound(s) is not likely to be a lipid because it appears to be hydrophilic. Nitration of proteins in the cells by NO_2, if any, also is unlikely because the UN-bearing compound(s) has a low molecular weight.

Fumigation of plants with NO_2 resulted in the accumulation of nitrite ion in leaf cells of

Arabidopsis thaliana plants. Similar results have been reported for pea, tomato, *Nicotiana glutinosa*, sunflower, kidney bean, spinach, and corn (Zeevaart 1976, Yoneyama and Sasakawa 1979, Yoneyama et al. 1979, Yu et al. 1988). At an acidic pH, nitrite ion reacts like nitrosonium ion (Beckman 1996) which also is highly reactive and forms nitroso compounds such as nitrosoamines with the amino acids and amines present in plant cells. Plant cells have substantial amounts of polyamines. Plant cells have substantial amounts of polyamines (Slocum 1991). The nitrosoamines formed between polyamines and nitrogen dioxide therefore are candidates for the UN-bearing compound(s). Consistent with this is the fact that preliminary analysis of the fraction 5 by GC/MS detected ^{15}N-bearing N-nitrosodiphenylamine (mol wt = 198) which accounted for about 10% of the UN (see above).

At a neutral pH, nitrite ion produces nitric oxide (NO) (Klepper 1979) and nitrous oxide (N_2O) (Porter 1969). An *in vivo* nitrate reductase assay, in which soybean leaf segments are suspended in a buffer, shows that a high concentration of nitrite ion results in the formation of NO and N_2O (Harper 1981, Dean and Harper 1986) and acetaldehyde oxime (Mulvaney and Hageman 1984). Some of these compounds also are poorly digested by the Kjeldahl method or are poorly reduced by Devarda alloy. Therefore they are possible candidates for the UN-bearing compound(s). The characteristics of the UN must be studied further in order to identify the UN-bearing compound(s) and its physiological function. Such information is important if we are to clarify the effects of this air pollutant on plant growth and the global environment. I currently are working on this, and results will be published elsewhere.

CONCLUSIONS

I have studied the role of GS isoenzymes in NO_2-assimilation pathway and the fate of NO_2 nitrogen in *Arabidopsis thaliana* leaves. My findings reported in the preceding chapters can be summarized as follows:

CHAPTER I

The cDNA clones for cytosolic and chloroplastic glutamine synthetase (GS1 and GS2, respectively) were isolated from cDNA library of *Arabidopsis thaliana* ecotype C24 and fully sequenced. The *gsR1* (GS1) and *gsL1* (GS2) cDNA clones contained, respectively, 1344 and 1556 nucleotide inserts, and each ORF translated into a 356 or 430 amino acid proteins. Three replaced amino acid was found in GS1 clone, compared with the cDNA for ecotype Columbia reported previously. Also, each ORF contained five kinds of the regions conserved in both prokaryotic and eukaryotic GS enzymes. ON the other hand, the genomic clones for GS1 and GS2 were isolated from *Arabidopsis thaliana* genomic library and fully sequenced. Compared with their cDNA sequences, each clone was concluded to be a GS1 gene composed by 9 exons and 8 introns or a GS2 gene composed by 11 exons and 10 introns, and they have a 1.4 and 1.2 kb of 5' flanking regions of the genes, respectively. In their 5' upstream region, we identified putative binding sites for NIT-2 and similar sequence motifs necessary for nitrate-inducible transcription of *A. thaliana* NR genes. These sequence motifs was also conserved in the 5' upstream region of GS and GOGAT genes from the other plant species.

CHAPTER II

Northern blot analysis showed changes of relative levels of *gsR1* (GS1) and *gsL1* (GS2) mRNAs in *Arabidopsis thaliana* leaves fumigated with 4 ppm NO_2 for up to 10 h. The GS1 mRNA level increased 2.5 times in response to fumigation with NO_2 1 h after the start of fumigation. Then it decreased and reached the initial level 10 h after the start. On the other hand, the GS2 mRNA level decreased drastically to 22% of the initial level 2 h after the start and increased to almost same level of the start for up to 10 h. This result suggest that NO_2 exhibit the different signals for gene regulations for GS1 and GS2 in plant leaves.

CHAPTER III

I report here an *in planta* transient expression assay using particle gun device, in which transgenes are introduced into the cells of leaves that are attached to an intact *Arabidopsis thaliana* plant. Using this method, a plasmid pSG containing 663-bp fragment of cDNA of the gene encoding GS in an antisense orientation under the control of cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthetase (NOS) polyadenylation signal was bombarded into the leaves attached to intact plants. Bombarded plants were subjected to standing in the atmosphere without added NO_2, or to fumigation with 4 ppm nitrogen dioxide (NO_2), and analyzed biochemically. Both GS enzyme activity and polypeptide levels in the leaves decreased when pSG-bombarded plants were stood in the atmosphere. Fumigation of pSG-bombarded plants with NO_2 much strengthened these decreases, and caused withering damages on their leaves. The analysis of the contents of nitrate, nitrite, and ammonium ions in the leaves revealed that the content of ammonium ions increased by 1.3-fold upon bombardment of pSG. Fumigation treatment of pSG-bombarded plants did not changed the

contents of NO_3^- or NH_4^+ ions but did increase (4-fold) the content of nitrite ion. This increase in the toxic ions was thought to cause damages of the leaves of pSG-bombarded plants upon fumigation. The present results provide evidence that GS is a key enzyme in the metabolism of NO_2 in *Arabidopsis thaliana* plants.

CHAPTER IV

Four kinds of plasmids that contain a full length or 3' UTR fragment of GS1 or GS2 cDNA in sense or antisense orientation under the control of CaMV 35S promoter and NOS polyadenylation signal were separately introduced into root sections of *Arabidopsis thaliana.* GS activity of T_2 lines containing GS2 cDNA in sense or antisense orientation varied greatly (the average value was respectively 39 to 208% of the control) in their leaves. On the other hand, those lines containing GS1 cDNA in sense or antisense orientation showed much lower GS activity (the average value was respectively 30 to 124% of the control) in their leaves. Western blot analysis showed some transgenic lines had overproduced and down-regulated GS1 and/or GS2 isozymes in their leaves.

CHAPTER V

I analyzed the excess ^{15}N levels in the total, reduced, ammonium, and nitrate/nitrite nitrogen (N) fractions from leaves of *Arabidopsis thaliana* plants fumigated with $^{15}NO_2$ or fed $K^{15}NO_3$. Lack of stoichiometry between the total N derived from NO_2 and the sum of (Kjeldahl) reduced and nitrate/nitrite N indicated that 20 to 30% of the total N is in the form of unidentified nitrogen (UN) in plants fumigated with $^{15}NO_2$. In contrast, there was stoichiometry between total N and the sum of reduced and nitrate/nitrite N in plants fed $K^{15}NO_3$. Forty to

60% of the nitrate-derived total N was found in the nitrate/nitrite N fraction, more than 10-fold the value for NO_2-derived nitrogen. A compound(s) bearing UN, formed before the conversion of NO_2 nitrogen to glutamine, that was recovered in the supernatant fraction of leaf symplasts was metabolically stable for at least a week. Ion exchange and gel permeation chromatography indicated that the UN-bearing compound(s) is cationic and of low molecular weight. Preliminary analysis by GC/MS detected ^{15}N-bearing N-nitrosodiphenylamine (mol wt = 198), which accounted for about 10% of the UN. These findings clearly indicate that the metabolic fate of NO_2 nitrogen differs from that of nitrate.

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