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Isolation of the spinach nitrite reductase gene promoter which confers nitrate inducibility on GUS gene expression in transgenic tobacco

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Abstract

Nitrite reductase is the second enzyme in the nitrate assimilatory pathway. The transcription of this gene is regulated by nitrate as well as a variety of other environmental and developmental factors. Genomic clones containing the entire nitrite reductase gene have been isolated from a spinach genomic library and sequenced. The sequence is identical in the transcribed region to a previously isolated spinach NiR cDNA clone (Back *et al.*, 1988) except for the presence of three introns. The analysis of the genomic clones and DNA blot hybridization demonstrates that there is a single NiR gene per haploid genome in spinach. This is in contrast to what has been found for other plant species. The transcription initiation site has been determined by S1 mapping and the 5′ upstream region has been used to regulate the GUS reporter gene in transgenic tobacco plants. This gene was found to be regulated by the addition of nitrate in the transgenic plants.

Introduction

Nitrate assimilation is, under most environmental conditions, the most important pathway by which plants can accumulate reduced nitrogen for incorporation into amino acids. Nitrate and nitrite reductase (NR and NiR) catalyze the two steps required for the assimilation of nitrate into ammonia. The expression of the genes coding for these enzymes is regulated primarily by the presence of nitrate, although there are a number of other environmental and developmental factors that modulate their expression. It has been shown for both NR [5, 7, 9] and NiR [1, 18] that the addition of nitrate leads to an increase in the steady-state level of mRNA which leads to a higher rate of protein synthesis. The other environmental stimuli that have been shown to modulate the expression of these genes include light [22, 25], water stress [15, 16], and the diurnal

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X17031.

cycle [3, 10, 12, 21]. Finally, these genes are regulated developmentally and are only expressed in certain cell types [13].

While a great deal of work has been done on the regulation of NR and NiR genes under different environmental conditions, the mechanisms underlying these phenomena remain unclear. This is in part due to the lack of higher-plant mutants altered for their regulation. Furthermore, the DNA sequences controlling expression of these genes have not yet been identified. One method that can be used to overcome these deficiencies is to analyze expression in transgenic plants. One can hopefully decipher the *cis*-acting elements responsible for the different types of regulatory control by mutating the 5′ upstream sequences. It also might be possible to use the expression of reporter or selectable genes under the control of defined promoter elements to isolate mutants altered in the regulation of the NR and NiR genes.

In this work, the analysis of genomic clones coding for spinach nitrite reductase is presented. Six independent clones were analyzed, with each being an overlapping piece from the same region of DNA. Along with DNA blot hybridization analysis, this demonstrates that there is a single NiR gene per haploid genome. The complete DNA sequence is presented and the introns and transcription initiation site are localized. Finally, a 3.1 kb upstream regulatory region was used to express a β-glucuronidase (GUS) reporter gene in transgenic tobacco plants, with its synthesis now being regulated by nitrate.

Materials and methods

Construction and screening of the genomic library

A genomic library was prepared as described in Maniatis *et al.* [19]. Genomic DNA was partially digested with *Sau* 3A and ligated into the *Bam* HI site of the λEMBL3 vector. Lambda DNA was packaged using the *in vitro* packaging extract from Promega. The library was screened for plaques which hybridized to the spinach NiR cDNA clone [1]. Out of 360000 screened plaques, 25 showed a positive signal. Six of the 25 plaques were isolated and a restriction map determined. All six clones contained overlapping DNA from the same genomic region. DNA sequencing [24] and computer sequence analysis were done as described previously [1].

S1 nuclease protection mapping and RNA blot hybridization

The end-labeled DNA probe was prepared in the following manner. pCIB403 plasmid DNA containing the *Bam* HI-*Pst* I fragment from the spinach NiR gene in pBSM13 was digested with *Pst* I and *Taq* I. From the resulting fragments, the 552 bp long *Taq* I-*Taq* I fragment was isolated using a low-melting-point agarose gel. The fragment was 5′-end-labeled using γ-^{32}P-ATP (6000 Ci/mmol; Amersham) and T4-polynucleotide kinase. After recleaving with *Bam* HI, the resulting 535 bp long *Bam* HI-*Taq* I fragment was isolated. Nuclease S1 mapping was carried out according to Berk and Sharp [2]. 20000 cpm of 5′-end-labeled DNA were mixed with 2.5 μg of poly(A)$^+$ RNA from spinach leaves [1] and 40 μg of tRNA from calf liver in 80% (v/v) deionized formamide, 0.4 M NaCl, 40 mM PIPES (pH 6.4) and 1 mM EDTA in a final volume of 25 μl. After heating to 70 °C for 15 min, hybridization was allowed to proceed at 39 °C either for 5 h or overnight. The samples were diluted 20-fold into ice-cold mung bean nuclease buffer (50 mM NaCl, 30 mM NaOAc pH 4.8, 1 mM $ZnCl_2$, 5% glycerol). Mung bean nuclease was added and the mixture was incubated at 37 °C for 50 min. The reaction was stopped by the addition of 50 μl stop buffer (5 M NH_4OAc, 8 mM EDTA, 200 μg/ml tRNA from calf liver), phenol-extracted and precipitated with isopropanol. Fragments were analyzed on a 5% sequencing gel. Total RNA was isolated as described [18] and electrophoresed through a 1.2% agarose, 2.2 M formaldehyde gel [19] and blotted onto nitrocellulose. The GUS gene was labeled using random primers from Pharmacia.

Construction of the NiR/GUS promoter fusion gene

A 3.3 kb DNA fragment which contained the NiR promoter sequences was subcloned into pBSM13. This DNA fragment spanned the region from the single *Pst* I site in the NiR coding region upstream to the *Sal* I site in which the NiR gene was originally cloned in the polylinker of the λEMBL3 vector. A *Bgl* II site was created by inserting the three bases TCT 13 bases before the initiating codon ATG using the *in vitro* mutagenesis kit Muta-Gene (Bio-Rad). The resulting clone was called NiR792. The *Sal* I-*Bgl* II fragment was then recloned into the plant transformation vector pBI101 [17] in the correct orientation to regulate the GUS gene (see Fig. 5).

Agrobacterium-*mediated plant transformation and plant cultivation*

Plant transformation experiments were done as described in Rothstein *et al.* [23]. The kanamycin-resistant plants were grown for approximately four weeks in GA-7 containers prior to transfer to sterilized potting soil and grown in growth chambers. The primary transformants were self-fertilized and the resulting seed collected. Plants from the F_1 seed were used in subsequent experiments. They were aseptically germinated on solid medium containing 0.5 × MS salts (pH 5.6), 1% sucrose, 0.8% agar, 400 μg/ml kanamycin. After two months seedlings resistant to kanamycin were transferred to soil/perlite and grown for an additional 2–4 weeks without fertilization. When the first leaf reached 10 cm in length, the plantlets were washed carefully and were grown for two weeks on a nitrate-minus medium (MS salts lacking either KNO_3 or NH_4NO_3, 20 mM KCl, 10 mM MES, pH 6.5) containing either 2 mM or 20 mM NH_4Cl was replaced with 20 mM KNO_3. The plant material was harvested, homogenized and the protein concentration determined, with an equal amount of protein used in the GUS assays. GUS activity was measured as described [17].

Results

Isolation of the gene coding for nitrite reductase from spinach

The gene coding for NiR was isolated from a genomic library of spinach DNA using a previously isolated NiR cDNA [1] as a probe. Restriction maps of six of these clones were prepared and proved to be overlapping clones isolated from the same region of genomic DNA. This finding is consistent with there being a single NiR gene per haploid genome. Figure 1 shows the physical map of the genomic NiR clone alignment when compared to the corresponding cDNA clone. Its entire DNA sequence was determined and is shown

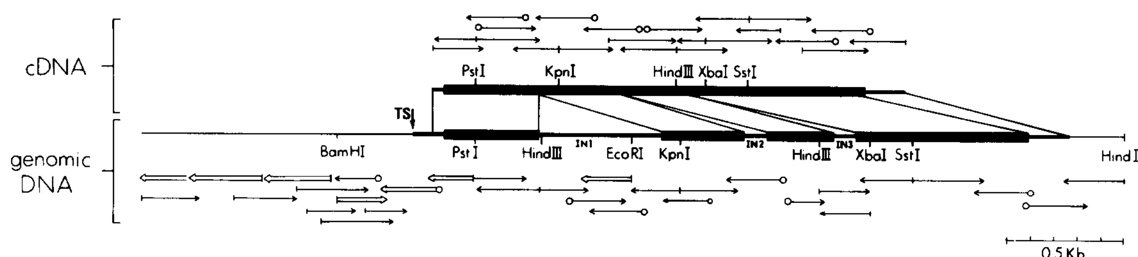

Fig. 1. Restriction map of the nitrite reductase gene from spinach. The wide bar represents the translated regions, the medium bar transcribed but untranslated regions and the narrow bar untranscribed regions. The coding region in the NiR gene is interrupted by three introns (In 1, In 2, In 3) of 531 bp, 93 bp and 93 bp, respectively. The transcription start site (TS) is marked by a vertical arrow. The NiR-cDNA clone in the upper half of the figure is from Back *et al.* [1]. The sequencing strategy is outlined by the arrows. A circle at the base of an arrow indicates where a synthetic oligonucleotide primer homologous to the genomic DNA has been used to prime the sequencing reaction. As a template for the sequencing reaction, either single stranded DNA (single arrow) or double-stranded DNA (double arrow) has been used.

in Fig. 2. The NiR gene coding region consists of 4 exons interrupted by three introns of 531 bp, 93 bp, and 93 bp, respectively. Each intron starts with the nucleotides GT and ends with AG, which are consensus sequences generally found in higher eukaryotes [4].

Localization of the transcription start site of the nitrite reductase gene

In order to determine the transcription initiation site, a mung bean nuclease protection assay was performed. The probe used was a 535 bp long *Taq* I-*Bam* HI fragment which was end-labeled with ^{32}P at the *Taq* I site which is located in the NiR coding region (see Materials and methods). It had previously been determined that NiR mRNA levels increased upon the addition of nitrate [1]. Therefore, a mung bean nuclease protection assay was done using poly$(A)^+$ RNA isolated from spinach plants grown in either the presence or absence of nitrate. A prominent, nitrate-inducible band of 204 nt was detected (Fig. 3). A number of fainter bands longer than 204 nt can be seen, but none of these are inducible with nitrate. Therefore, the 204 nt band is the most likely start site of transcription. Three nitrate inducible bands that are slightly shorter can also be seen in Fig. 3. These may represent secondary transcription start sites or may be due to degradation of the RNA. It is also possible that the larger, non-nitrate-inducible bands represent constitutive transcripts since there is a low level of NiR mRNA present in the leaves in the absence of nitrate [1, 18).

The exact start site of transcription was determined at the nucleotide level by gel electrophoresis of the mung bean products next to a Maxam and Gilbert [20] sequencing ladder. As can be seen in Fig. 3, the transcription of the NiR gene starts with a G (a C in the non-coding strand on the sequencing gel). This site is notated as position +1 on the DNA sequence shown in Fig. 2. There is a TTATTA sequence 25 bp upstream that is correctly located to position the RNA polymerase to initiate transcription at the correct site although the sequence differs somewhat from plant consensus sequences for the TATA box [11]. There is a CAAAAT sequence at position −80. This is also present in a similar location in the two NR genes from *Nicotiana tabacum* [26]. It is unclear at this time whether this sequence serves an important function.

One gene coding for spinach nitrite reductase is present per haploid genome

In order to determine the copy number of the NiR gene in spinach, genomic DNA was digested with either *Bam* HI, *Eco* RI or *Hin*d III. DNA blot hybridization analysis of the DNA using the spinach NiR cDNA clone as a probe is shown in Fig. 4. All the bands visible on the autoradiograph are as expected given the restriction pattern of the cloned NiR gene. Several other restriction enzymes which do not cleave within the gene give a single band (unpublished results). This result makes it extremely likely that there is only one NiR gene per haploid genome, since any other copy of the gene would need to have introns of the same size, as well as similar flanking DNA sequences. This is supported by the observation that all six independently isolated genomic clones are over-lapping clones from the same region of chromosomal DNA.

Nitrate inducibility of the NiR promoter fused to the GUS gene in transgenic tobacco plants

The upstream regulatory elements from the NiR gene were isolated as a 3.3 kb *Sal* I-*Pst* I fragment which contains a small portion of the coding sequence. A *Bgl* II site was inserted just upstream of the translation start. The resulting 3.1 kb *Sal* I-*Bgl* II fragment was cloned into pBI101.2 [17] so that the transcription of the GUS gene would initiate from the NiR promoter (Fig. 5). This construct was transformed into tobacco plants using kanamycin resistance as the selectable marker. The initial transformed plants were allowed to self-pollinate and their seeds germinated on

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-1164       ATAGAAGGGTGACAAAGTGATAGAATTGCAAGGGGTAGGGGAAGACCTAAGAAAACTTGGAGGAAGGTGATTGA
-1090  ACACGATATAAGGTTTCTTGGGATTGAGGAAAATATGGCGTTGGATAGGACAGAGTGGAGAGAGTTCATTTGACTTATAC
-1010  AACTTTCATGTTTCTGTTTCTTTCTATTTTTTATTTTATTTTTTATTCTTTTTTTTATTTTTTACTTTTTTTAAAAAAAT
 -930  TCCTTTATTTTTATTTTTTTCTTACATGCTATTTGAAATGTACTTATTTTACTTATTCGTTTATTTTAAACTTTACTTAT
 -850  TTTTTGTTATCCCTTACAATATTTCTTCTATAATATATATACATTTTTCTCTTATCTTACTTTCATTAATTCCATTTTTT
 -770  CTTCCTTGTTTTTTATCTTTTTAACTTCCTGCTCATTTCTGGTTTGATTGGTGACAATGACGATCTCCTACGACAATTCA
 -690  TGTTAGCCGACCCCAAATCATTACTAAGGCTTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGT
 -610  TGTTGTTGTATCTGAACGGGCAAACCCAACCTATCATAACACACCCGACCTAGCTAAGCTCGCGACCTACTTAATGTACG
 -530  TAGACATCCTAACATAGTTGACCTTTCCCGAACCACCCTAATCCTTAGTTGTATTGGTTGAGGTCACCCAACTCTTGCGA
 -450  CCTACCTAACTTGGCACAGATACACCCGCCACAATGATTATCTCCAGTTTAAGAATCCTGGACAATCTATCACTAAAATA
 -370  CACATATCAATTAGAGTTATATTTCACAGAAAAGGATATCGGATCCGAGATTTGAAATGAATGCCATTGATTTCAATTTA
 -290  TCAGTCCTTTAACATTAAATCAAAACCTAGTTAGTTTTTCATACATACATTGATCAATTTTATGCAAAGCGACAAAAATA
 -210  GATATTAGTAACACAACATTACATATTATATCTAGCACCATACTATAATGGTTGGCGGCTAGAGGCAGTCTGCCCTTTTA
 -130  GCCGCTAGTTTTGGGTGTGAATGGCCATCCAACGTAACCAAACATACAAAATGACCCTTAACCATGTCCAAGAGTCCCCT
  -50  CTTAACTCTTCCCACCTTGTGCTTATTACTAGTTTCCACACTCCCTCACCGTTTGTCCTTCTCCTCTTTCTCTCTCCTCA
   30  ATCACCCTACATAAAAATACAATTTCAATTCCACCACTAACTCATCATCATCATCATCATCTTCATCTTCATCTTCATCA
  110  TTCATAGTTGCAAGAAACAGAGCAACCAAAAAAAATGGCATCACTTCCAGTCAACAAGATCATACCATCATCAACGACAT
                                           MetAlaSerLeuProValAsnLysIleIleProSerSerThrThrL

  190  TACTGTCATCGTCGAACAACAACAGAAGAAGAAATAACTCATCAATTCGATGCCAGAAGGCGGTTTCACCCGCGGCAGAA
       euLeuSerSerSerAsnAsnAsnArgArgArgAsnAsnSerSerIleArgCysGlnLysAlaValSerProAlaAlaGlu

  270  ACGGCTGCAGTGTCGCCGTCTGTGGACGCGGCGAGGCTGGAGCCGAGAGTGGAGGAGAGAGATGGGTTTTGGGTATTGAA
       ThrAlaAlaValSerProSerValAspAlaAlaArgLeuGluProArgValGluGluArgAspGlyPheTrpValLeuLy

  350  GGAGGAATTTAGGAGTGGGATTAACCCAGCTGAGAAAGTTAAGATTGAGAAAGACCCAATGAAGTTGTTTATTGAGGATG
       sGluGluPheArgSerGlyIleAsnProAlaGluLysValLysIleGluLysAspProMetLysLeuPheIleGluAspG

  430  GGATTAGTGATCTTGCTACTTTGTCAATGGAGGAAGTTGATAAATCTAAGCATAATAAGGATGATATTGATGTTAGACTC
       lyIleSerAspLeuAlaThrLeuSerMetGluGluValAspLysSerLysHisAsnLysAspAspIleAspValArgLeu

  510  AAGTGGCTTGGACTTTTCCATCGCCGTAAACATCACTGTAAGCTTAACTAACTTCCTAATTGTTGTTGTATAATTCGATT
       LysTrpLeuGlyLeuPheHisArgArgLysHisHisT

  590  TTTTTTTAAAAGATTAGATGATTAATTAGATCCATTTTTATGATTGGTTTTGATTCAATTTAAGAGGTTAATTGCTTGAT
  670  TAGGAGATATTTTCTGTTGGTTAGTCAACGAATTTCATGTCGCAATCGCTTAAAAGGGATGTTGTTAGTATAATCGTACG
  750  TAATTAGCTTGTTAATTTAATCATTGAATATGGTTTTTATATCGTTTTGCTGAAAATTCGAAACCCAGAAATCGGATTGA
  830  ATATTGATGATTGAGGAATATGGTTAGAAGAAGTTATAGTTGAAATTGTAATTAGCTTAGTAATTGGATCATTTCATTAA
  910  ATTTGAAATCCCGGAATCGGACTGAATATGGAGGAATTCCGTAGTAAAACTGTAGCTACTGGACTCAGTAGAAATGAATT
  990  GTCAGTATGATTGATGGATAATGTTAAATGTTTATGTAATGGAGTGAGTCTGATGGATGACTGTAAAAAATGGTGGATGT
 1070  TATTAGATGGGAGATTCATGATGAGGTTGAAGCTGCCGAATGGGGTAACAACGAGTGAGCAGACACGGTACCTAGCAAGC
             yrGlyArgPheMetMetArgLeuLysLeuProAsnGlyValThrThrSerGluGlnThrArgTyrLeuAlaSer

 1150  GTGATCAAGAAGTACGGAAAAGATGGATGTGCGGATGTAACAACAAGGCAAAACTGGCAAATTAGAGGAGTTGTTCTGCC
       ValIleLysLysTyrGlyLysAspGlyCysAlaAspValThrThrArgGlnAsnTrpGlnIleArgGlyValValLeuPr

 1230  TGATGTGCCAGAGATCATCAAAGGGCTGGAATCCGTTGGTCTTACCAGCTTACAGAGTGGGATGGACAATGTAAGGAACC
       oAspValProGluIleIleLysGlyLeuGluSerValGlyLeuThrSerLeuGlnSerGlyMetAspAsnValArgAsnP

 1310  CTGTAGGTAACCCTCTTGCAGGGATTGACCCTCATGAAATTGTTGACACCCGACCTTTTACCAACCTAATTTCCCAATTT
       roValGlyAsnProLeuAlaGlyIleAspProHisGluIleValAspThrArgProPheThrAsnLeuIleSerGlnPhe

 1390  GTCACTGCCAATTCGCGTGGAAACCTTTCTATTACCAATCTGTAAGTCCTTTCGGTATCTCTTTCAAGCATGTTATGGTA
       ValThrAlaAsnSerArgGlyAsnLeuSerIleThrAsnLe

 1470  AATCTGTATTAGTAACTTGTTAGGCGCTGTTGTTTGTTTTGAACATTGGTTCAGGCCAAGGAAGTGGAATCCATGTGTTA
                                                             uProArgLysTrpAsnProCysValI

 1550  TTGGGTCCCATGATCTTTATGAGCATCCACACATCAATGACCTTGCTTACATGCCTGCTACAAAGAATGGGAAATTCGGG
       leGlySerHisAspLeuTyrGluHisProHisIleAsnAspLeuAlaTyrMetProAlaThrLysAsnGlyLysPheGly

 1630  TTTAATTTGTTGGTTGGAGGATTCTTTAGCATCAAAAGATGTGAAGAGGCAATCCCACTAGACGCTTGGGTCTCAGCAGA
       PheAsnLeuLeuValGlyGlyPhePheSerIleLysArgCysGluGluAlaIleProLeuAspAlaTrpValSerAlaGl

 1710  AGATGTGGTTCCTGTATGCAAAGCTATGCTTGAAGCTTTCAGGGACCTTGGCTTTAGAGGAAACAGGCAGAAGTGCAGAA
       uAspValValProValCysLysAlaMetLeuGluAlaPheArgAspLeuGlyPheArgGlyAsnArgGlnLysCysArgM

 1790  TGATGTGGCTTATTGATGAGCTTGTGAGTACTACTAACAAACAACTCTCCTCTTACTAGTTAATCTATTCTAAGTAATTA
       etMetTrpLeuIleAspGluLeu

 1870  TTCTAACTGTATTGCTCATTCTCCAAACAATGGCAGGGTATGGAAGCATTCAGGGGAGAGGTTGAGAAGAGAATGCCTGA
                                             GlyMetGluAlaPheArgGlyGluValGluLysArgMetProGl

 1950  GCAAGTTCTAGAAAGAGCATCCTCAGAAGAGCTGGTTCAGAAGGACTGGGAGAGAAGAGAATACTTAGGAGTTCACCCTC
       uGlnValLeuGluArgAlaSerSerGluGluLeuValGlnLysAspTrpGluArgArgGluTyrLeuGlyValHisProG

 2030  AGAAACAACAAGGACTTAGCTTTGTGGGTCTCCACATTCCTGTGGGCCGTCTGCAAGCTGATGAGATGGAAGAGTTAGCC
       lnLysGlnGlnGlyLeuSerPheValGlyLeuHisIleProValGlyArgLeuGlnAlaAspGluMetGluGluLeuAla

 2110  CGTATAGCTGATGTGTATGGATCAGGGGAGCTCCGTCTGACAGTAGAGCAGAACATAATCATCCCAAATGTTGAAAACTC
       ArgIleAlaAspValTyrGlySerGlyGluLeuArgLeuThrValGluGlnAsnIleIleIleProAsnValGluAsnSe

 2190  AAAGATAGATTCACTACTAAACGAGCCTCTGTTAAAAGAGCGTTACTCCCCTGAACCACCCATCTTGATGAAGGGGCTTG
       rLysIleAspSerLeuLeuAsnGluProLeuLeuLysGluArgTyrSerProGluProProIleLeuMetLysGlyLeuV

 2270  TGGCCTGTACGGGGAGCCAATTTTGTGGACAAGCCATTATCGAGACCAAGGCTAGGGCACTCAAGGTGACAGAAGAGGTA
       alAlaCysThrGlySerGlnPheCysGlyGlnAlaIleIleGluThrLysAlaArgAlaLeuLysValThrGluGluVal

 2350  CAACGACTAGTGTCTGTAACACGGCCTGTTAGGATGCATTGGACCGGGTGTCCTAATAGTTGTGGTCAAGTACAAGTGGC
       GlnArgLeuValSerValThrArgProValArgMetHisTrpThrGlyCysProAsnSerCysGlyGlnValGlnValAl

 2430  TGATATTGGGTTCATGGGTTGCATGACTAGGGATGAGAACGGTAAGCCTTGTGAAGGAGCTGATGTGTTTGTAGGAGGAC
       aAspIleGlyPheMetGlyCysMetThrArgAspGluAsnGlyLysProCysGluGlyAlaAspValPheValGlyGlyA

 2510  GTATAGGAAGTGACTCGCATCTAGGAGACATTTACAAGAAGGCAGTCCCATGTAAAGATTTGGTGCCTGTTGTTGCTGAG
       rgIleGlySerAspSerHisLeuGlyAspIleTyrLysLysAlaValProCysLysAspLeuValProValValAlaGlu

 2590  ATATTGATCAACCAATTCGGTGCTGTTCCTAGGGAGAGGGAAGAGGCAGAGTAGTAGCTAGACTGTTTTGGGTGCCTGTT
       IleLeuIleAsnGlnPheGlyAlaValProArgGluArgGluGluAlaGluEnd

 2670  CTTGTTAACTGTTATCGGTATTCGGTAATTACTTGTAATATTTGCATTTTTTTTCAAGCATATAATTAAATTGCATAAAG
 2750  ATCCCTTGTATGTCTGCATAACAAGATACTCAGTTATGTAATGTCAATAGCAGGTTTACTTTGTTTATTCAATAGGCACT
 2830  GTGAAAGGGAAAGTTCATTATTCATTTCTCACAATGTTTCCAATTTGAGATCGAAAAAATATATATAATATTGTCTACAT
 2910  CATTTACGGTATTGGAACGTTCGCTACAGAAAAAAAGAAAGTTGACTTGATCATTTGTTATCATATCTAAATTTCAACAT
 2990  ATCGCTATCTGTCTTCGAAAGTAAAGATGCGAAACCATCAGCAGAGAGGCAATTCAGGCAAACCAGCATTCAAGAAGCTT
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Fig. 2. DNA sequence and deduced amino acid sequence of the NiR gene from spinach. Transcription starts with the nucleotide G (underlined) at position + 1. A TATA-like sequence (TTATTA) is found around base pair − 25 and a CAAT-like sequence (CAAAAT) around base pair − 80.

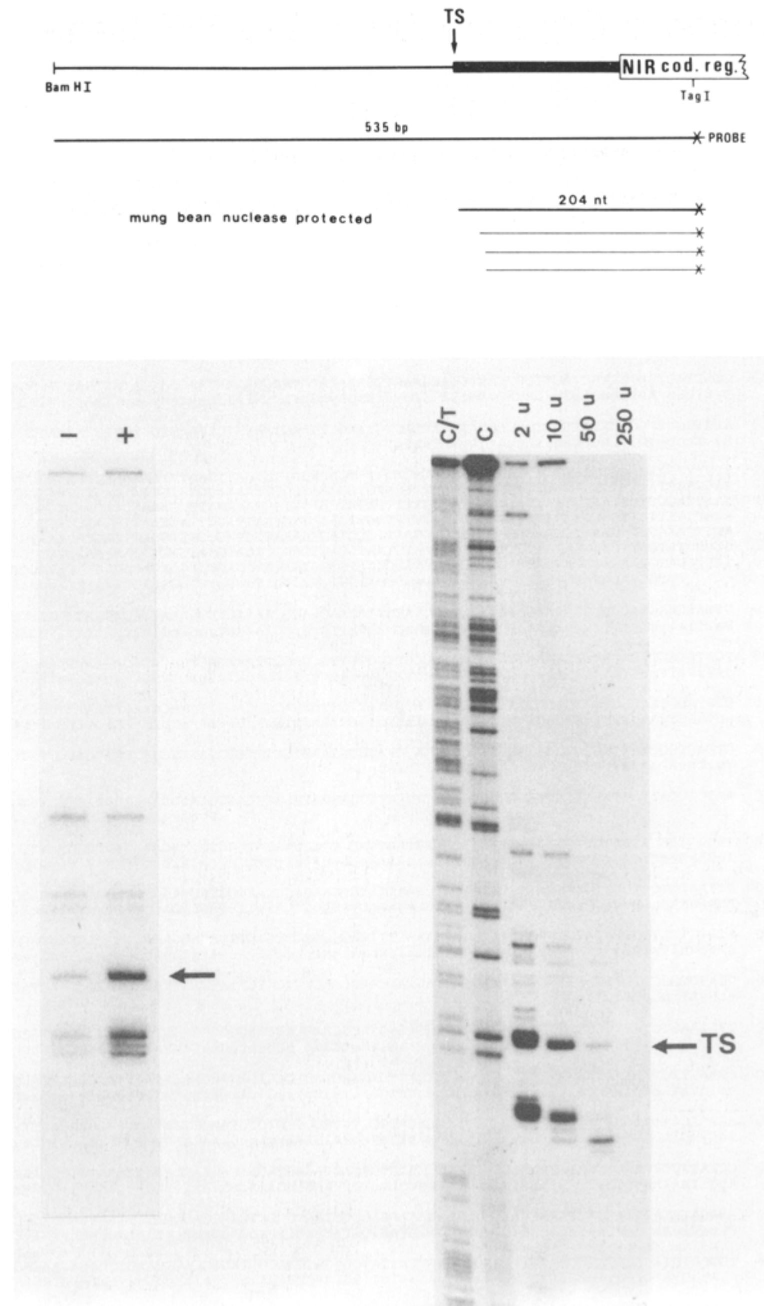

Fig. 3. Localization of the transcription start site of the NiR gene. Top: a schematic drawing of the mung nuclease protection experiment. Bottom left: a mung bean nuclease protection experiment using poly(A)$^+$ RNA isolated from leaves of plants that had either been induced (+) or not induced (–) with nitrate. The transcription start site corresponds to the longest nitrate inducible and mung bean protected band of about 200 nt in length and is denoted with an arrow. Bottom right: DNA fragments resistant to 2, 10, 50 or 250 units of mung bean nuclease were electrophoresed next to a Maxam and Gilbert [20] sequencing ladder prepared from the probe used in the protection assay. The transcription start site (TS) is the nucleotide C which corresponds to a G in the coding strand.

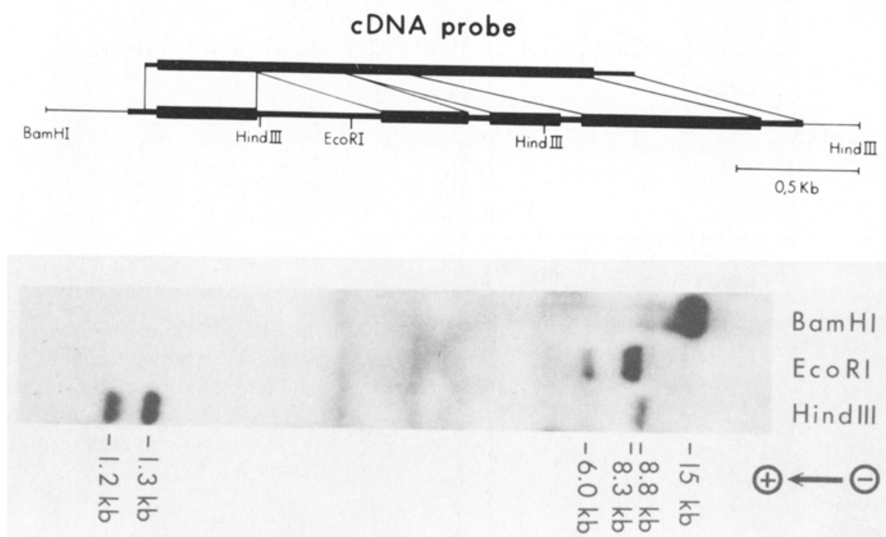

Fig. 4. Determination of the nitrite reductase gene copy number in the spinach genome. The schematic drawing at the top of the figure illustrates the expected restriction fragments if the NiR gene is digested by the indicated restriction enzymes. The bottom of the figure shows a DNA blot hybridization in which digested spinach leaf DNA is probed with the radioactively labelled spinach NiR cDNA clone.

medium containing kanamycin. The kanamycin-resistant plants were then analyzed for GUS activity as described below. As a control, a plant transformed with the GUS gene, but lacking a promoter, was analyzed (a gift from Dr Josef Bichler, Munich).

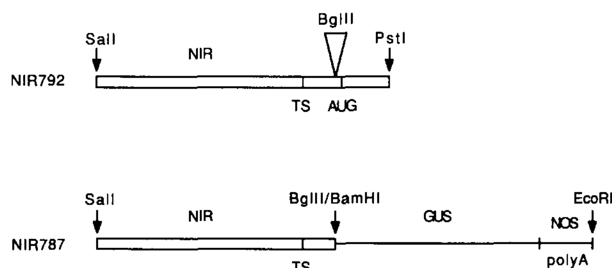

Fig. 5. The NiR promoter-GUS reporter gene plasmid construct. The spinach NiR 5′ upstream regulatory sequences were inserted into pBSM13 between the *Sal* I and *Pst* I restriction sites. A *Bgl* II site was inserted into this construct by *in vitro* mutagenesis just upstream of the translation initiation site making NiR792. The resulting *Sal* I-*Bgl* II fragment was then inserted upstream from the GUS gene in the plasmid NiR787. This plasmid was used to transform tobacco plants via *Agrobacterium.* TS, transcription start site; AUG, translation start site.

The resistant plants were transferred to a soil/perlite mix and when the largest leaf reached 10 cm in length they were washed carefully and watered for two weeks with a mixture in which the sole nitrogen source was 2 mM ammonium. At this time, the plants were washed again and fertilized with a solution containing 20 mM KNO_3. For each plant, leaf and root material was harvested at 0 time (just prior to the addition of nitrate) and then 48 and 120 hours after nitrate was added. The leaf material used was from leaf '4' which was 15 cm long. The results of the GUS assays are shown in Fig. 6 for plants derived from four different primary transformants. The amount of GUS activity increases upon the addition of nitrate in both roots and leaves. On average, the GUS activity in roots increased by approximately five-fold, while in leaves the increase was three- to four-fold. Furthermore, the total amount of GUS activity per mg total protein was considerably higher in roots than in leaves. There is a consider-

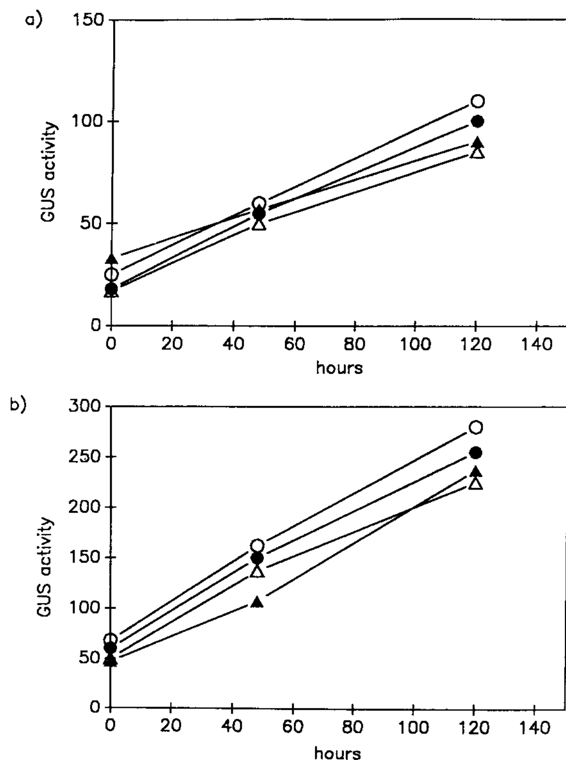

Fig. 6. Analysis of tobacco plants transformed with the NiR promoter-GUS construct. Plants were grown with ammonia as the sole nitrogen source until time 0. At that point, the plants were watered with a solution containing 20 mM nitrate. Leaf and root samples were collected from each transformed line at time 0 (prior to the addition of nitrate) and then 48 h and 120 h after the addition of nitrate. The samples were assayed for GUS as described in Material and methods. 1000 units of GUS activity is the amount of enzyme that produces 980 nmol of methylumbelliferone per mg protein per minute. Each tissue aliquot came from five plants with the experiment being repeated twice with identical results. a) analysis of GUS activity in leaves; b) analysis of GUS activity in roots.

able constitutive level of GUS activity in these plants prior to the addition of nitrate. However, when mRNA levels were analyzed in one of the transformed lines, there was only a low level of constitutive GUS mRNA while the addition of nitrate led to a marked increase in mRNA level (Fig. 7). Therefore, the presence of fairly high levels of constitutive GUS activity is most likely due to the stability of the protein once it is synthesized.

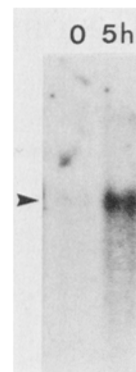

Fig. 7. Induction of GUS mRNA by nitrate in a transgenic tobacco plant line harboring the NiR promoter-GUS construct. Plants were induced as described in Fig. 6, and leaf samples harvested at time 0 and 5 h after the addition of nitrate.

Discussion

The gene for spinach NiR was cloned and sequenced. The sequence exactly matches that found earlier for the corresponding cDNA clone [1] except for the presence of three introns. Six different genomic clones were analyzed and found by restriction analysis to be overlapping clones from the same chromosomal region. The simplest explanation of these results, along with the DNA blot hybridization analysis, is that there is only one NiR gene per haploid genome. In contrast, it has been found that there is more than one NiR gene in maize per haploid genome ([18] and our unpublished results). It has also been demonstrated that more than one NiR isoenzyme can be found in several species ([14] and references within). Therefore, there is certainly a diversity with respect to the number of NiR genes amongst plant species.

The expression of the NiR gene is regulated at the level of transcription by the addition of nitrate. Since, NR is also regulated by nitrate, one might expect that there is a similar regulatory element within the promoter regions of these genes that is responsible for nitrate induction. A computer comparison of the spinach NiR gene and the *N. tabacum* NR genes [26] does not reveal any striking sequence homology (M. Caboche, per-

sonal communication). The only similar sequence is a CAAAAT sequence 80 bp from the transcription start site in the spinach NiR gene which is in a virtually identical location in the NR genes. It is therefore difficult to predict by sequence comparison what region might be important for regulation of these genes by nitrate.

The NiR upstream regulatory sequences were placed adjacent to the GUS reporter gene and transformed into tobacco cells via *Agrobacterium*. Regenerated plants were analyzed for the expression of GUS in leaves and roots. Given that there is only a single spinach NiR gene and that the NiR protein is found both in leaves and roots, one would expect that GUS would be expressed in leaves and roots in these plants. Most importantly, one would expect the amount of enzyme activity to be regulated by nitrate in both tissues. This indeed did turn out to be the case. Under the conditions used, the amount of GUS activity increases approximately 5-fold in roots and 3- to 4-fold in leaves upon the addition of nitrate. This level of induction was very similar to that found when the spinach NiR gene expression was analyzed in plants grown under similar conditions [1]. While there was a considerable level of GUS activity prior to the addition of nitrate, this is most likely due to the presence of low levels of nitrate present in the soil of the plants due to ammonium oxidation by microorganisms. In that case, one would expect that the actual level of induction in plants grown under sterile conditions would be much higher. The level of GUS mRNA for the transformed line tested was shown to increase markedly upon the addition of nitrate demonstrating that, as one would expect, the nitrate induction of GUS activity was due to changes in the GUS mRNA level.

The nitrate inducibility of the GUS reporter gene demonstrates that the regulatory elements required for this process are in the upstream regulatory regions as one might expect. In the filamentous fungi, the regulation of the nitrate assimilatory genes has been delineated in detail due to the isolation of large numbers of mutants [8]. Even though a number of mutants have been isolated in the genes coding for the NR apoenzyme and those involved in the biosynthesis of its prosthetic group ([6] reference therein), none have been found that specifically alter the regulation of NR and NiR expression. It will hopefully be possible to identify the regulatory regions that are crucial for nitrate regulation through the expression of reporter genes in transgenic plants. Furthermore, it might be possible to isolate mutants altered for the expression of a reporter gene regulated by the NiR promoter. If that were possible the same approach that has yielded so much information in the fungal systems could be used to study these genes in plants.

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