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A 330 bp region of the spinach nitrite reductase gene promoter directs nitrate-inducible tissue-specific expression in transgenic tobacco

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Summary

Nitrite reductase is an enzyme in the nitrate assimilatory pathway whose expression is induced upon the addition of nitrate. Furthermore, it is known to be located in chloroplasts in leaves and plastids in roots. A 3.1 kb 5′ upstream region of the spinach nitrite reductase (NiR) gene promoter was shown previously to confer nitrate inducibility on the β-glucuronidase (GUS) reporter gene expression in both the leaves and the roots of transgenic tobacco plants. In the present study, this 3.1 kb promoter fragment as well as a series of promoter deletion constructs, fused to a GUS gene, were utilized to delineate the region of NiR promoter involved in nitrate regulation of NiR expression by studying the cellular localization of NiR–GUS expression as well as its regulation by nitrate. In plants carrying the longest promoter fragment (−3100 from the transcription start site) and promoter sequences progressively deleted to −330 bp, the expression of GUS was markedly increased in the presence of nitrate, and this expression was found to occur in mesophyll cells in leaves and in the vascular tissues of stem and roots. When nitrate was added to NiR–GUS plants grown in the absence of nitrate, significant levels of GUS activity could be seen in the roots after 2 h and in the leaves after 6 h. Further 5′ deletion of the promoter to −200 bp abolished the nitrate induction of GUS expression, indicating that the 130 bp region of the nitrite reductase promoter located between −330 and −200 is required for full nitrate-inducible tissue-specific expression.

Introduction

Under normal field conditions, nitrate is the most common source of inorganic nitrogen used by higher plants. After being transported into cells, it must be reduced to ammonium which is then incorporated into amino acids. The reduction of nitrate to ammonium is catalyzed by two enzymes: nitrate reductase (NR) which reduces nitrate to nitrite and nitrite reductase (NiR) which reduces nitrite to ammonium. The expression of the genes encoding NR and NiR is regulated by a variety of factors such as the presence of nitrate (Back *et al.*, 1988; Cheng *et al.*, 1986; Crawford *et al.*, 1986; Lahners *et al.*, 1988), light and diurnal cycle (Bowsher *et al.*, 1991; Deng *et al.*, 1990; Galangau *et al.*, 1988) growth hormones (Lu *et al.*, 1990, 1992) and nitrogen metabolites (Deng *et al.*, 1991). Nitrate, however, appears to be the most important regulator of the expression of NR and NiR, with its addition to the plants leading to a marked increase in the steady-state levels of NR and NiR mRNAs (for reviews see Caboche and Rouze, 1990; Crawford and Campbell, 1990; Solomonson and Barber, 1990).

The reduction of nitrate in higher plants takes place primarily in the leaves and/or the roots, although almost all tissues investigated have the capability of producing enzymes required for nitrate assimilation (Andrews, 1986; Guerrero *et al.*, 1981). While it is known that nitrate assimilation occurs in mesophyll cells in the leaf, the distribution of the nitrate assimilatory enzymes in other cell types is not well documented. The intracellular localization of NR remains a matter of controversy although most evidence suggests a cytoplasmic location (Solomonson and Barber, 1990). However, NiR has been localized to the chloroplasts in leaves and the plastids in non-green tissues (Guerrero *et al.*, 1981). The plastidic location of NiR is further confirmed by the presence of a transit peptide in the spinach NiR precursor protein governing its transport (Back *et al.*, 1988).

While considerable work has been done in higher plants on the regulation of expression of NR and NiR genes by different factors, very little is known of the underlying molecular mechanisms. On the other hand, in filamentous fungi (*Neurospora* and *Aspergillus*), there is a better understanding of the regulation of the nitrate assimilatory genes. Their expression requires simultaneous nitrogen derepression and nitrate induction, and is governed by positive regulatory genes, namely the *areA* and the *nit-2* genes of *Aspergillus* and *Neurospora*, respectively (Marzluf,

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1981). These genes code for *trans*-acting proteins that turn on the expression of various structural genes involved in nitrogen metabolism including nitrate reductase (Fu and Marzluf, 1990; Kudla *et al.*, 1990). In higher plants, no analogous regulatory genes have yet been identified.

There is some information regarding the *cis*-acting elements that are involved in the regulation of expression of the NR and NiR genes in higher plants. For example, in an earlier paper we showed that a 3.1 kb 5′ upstream region of the spinach NiR gene confers nitrate inducibility on β-glucuronidase (GUS) gene expression in transgenic tobacco (Back *et al.*, 1991). Cheng *et al.* (1992) have recently reported that a 2.7 kb region of the 5′ flanking sequences of the *Arabidopsis* NR gene is sufficient to confer light and sucrose induction of the chloramphenicol acetyltransferase reporter gene in transgenic *Arabidopsis* plants. Also, a 3 kb 5′ upstream region of the tomato *nia* gene was shown to be sufficient for regulation of NR expression by nitrate, light and circadian rhythm when it was introduced into a NR-deficient mutant of *Nicotiana plumbaginifolia* (Dorbe *et al.*, 1992). However, there is no information on the DNA sequences involved in the regulation of the nitrate assimilatory genes by various stimuli.

In this study, a series of deletions in the spinach NiR promoter, fused to a GUS gene, were analyzed for their effect on the pattern of GUS gene expression in various tissues and on the induction of this expression by nitrate. The −330 region of the NiR promoter appears to contain all of the required *cis*-acting elements regulating the expression of this gene. However, when the −200 bp fragment of the upstream region is present, no expression of the GUS gene is seen implying that the region of the NiR promoter between the 330 and 200 bp upstream of the transcription initiation site is crucial for NiR gene expression.

Results

The sequence, structure and organization of the spinach NiR gene were described previously (Back *et al.*, 1991). It was also shown that a 3.1 kb fragment of the NiR promoter can direct nitrate-regulated GUS expression in the leaves and the roots of transgenic tobacco plants, and that the induction of GUS activity by nitrate was due to changes in the GUS mRNA level. In order to delineate the regions of the NiR promoter involved in regulation by nitrate, a series of deletions were made in the 5′ upstream region (Figure 1), fused transcriptionally with a GUS gene, and the chimeric NiR–GUS constructs transformed into tobacco. The deletions differed only in promoter length, and all other features were identical for the various constructs. For each construct, 9–13 independent transformants were analyzed for regulation of the NiR–GUS fusion gene by nitrate.

Effects of 5′ promoter deletions on nitrate induction of GUS activity

In the previous work (Back *et al.*, 1991), the NiR 787 plants were grown for 2 months in the presence of nitrate and were then grown in the absence of nitrate for 2–4 weeks

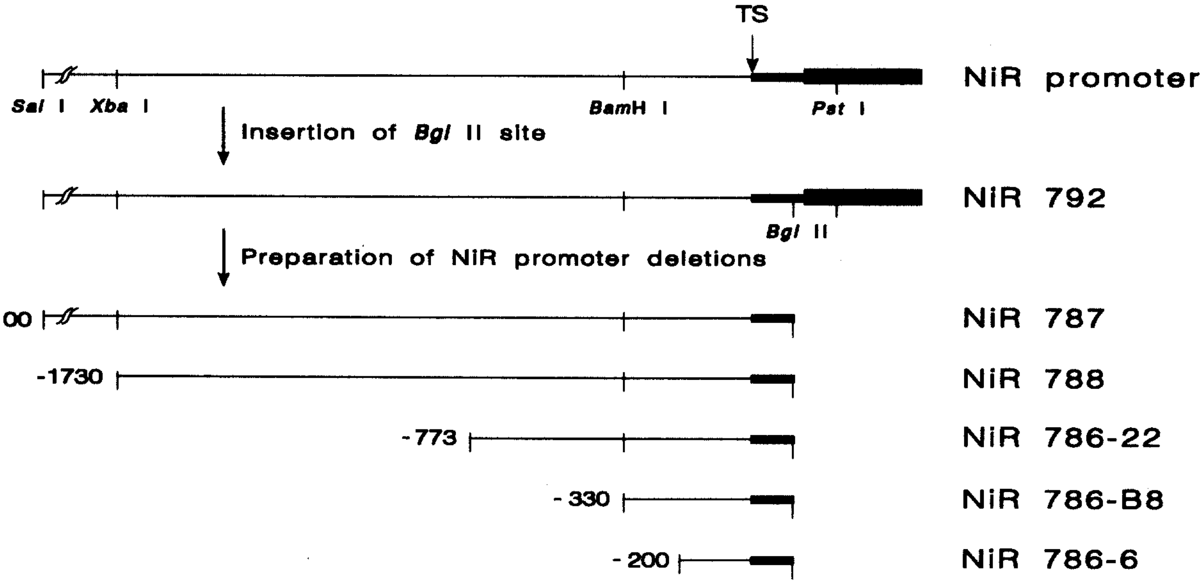


Figure 1. Schematic representation of 5′ deletions of the NiR gene promoter.
The 5′ ends of the deletions, relative to the transcription start site (TS), are indicated. The truncated promoter fragments were inserted in the correct orientation upstream of the GUS gene in the polylinker region of pBI101, and the resulting plasmids were used to transform tobacco.

prior to nitrate induction. In such plants, a considerable level of GUS activity was observed even in the absence of nitrate, most likely due to residual nitrate or traces of nitrate produced from ammonium oxidation. In order to maximize the level of nitrate induction, tobacco seedlings in these experiments were grown aseptically using either nitrate or ammonium as the nitrogen source. For the initial screening, GUS histochemical assays were performed on the leaves and the roots of transformants grown aseptically on these two media. The majority of the transformants with the various promoter constructs, except 786–6, showed an increase in GUS activity in the presence of nitrate in both leaves and roots (Table 1) as determined by the increase in the intensity of the blue color. There were, however, variations among the independent transformants of a given construct with respect to basal GUS activity and the magnitude of nitrate-regulated increase. In two of the transformants (one each of 787 and 786-B8), the level of GUS activity was not influenced by nitrate (Table 1). None of the 10 independent transformants of 786-6 (with the −200 bp fragment of the promoter) showed any detectable GUS activity in the roots or the leaves, either in the presence or the absence of nitrate.

For each construct, three to five independent transformants were analyzed in detail for GUS activity measurements (Table 2) and tissue localization. GUS activity was measured in leaf and root tissues of transgenic plants grown on ammonium- and nitrate-containing media. Variations in basal and nitrate-increased GUS activity, as noted earlier, were observed among the transformants of a given construct, and therefore, the data were not pooled. It should also be noted that in most transgenic lines GUS specific activity in the roots was higher than that in the leaves (Table 2). However, when expressed on a unit fresh weight basis, GUS activity was similar in the two organs (data not shown). Most of the transformed lines carrying the NiR promoter between 3.1 kb and 330 bp upstream of the transcription initiation site showed little or no GUS activity when grown on the ammonium-containing medium, but in the presence of nitrate, a large increase in GUS activity was observed in the leaves and the roots (Table 2). In one transformant of 786-B8, which in the initial screen showed no nitrate induction of GUS activity, GUS activity was not affected by the presence of nitrate in the medium (Table 2). The deletion construct containing only 200 bp of the NiR promoter upstream of the transcription start site gave no measurable GUS activity in the presence or absence of nitrate (Table 2). This abolition of promoter activity was not due to loss or rearrangement of the transgene as confirmed by DNA blot hybridization (data not shown). It therefore appears that the region of the NiR promoter between −330 and −200 contains regulatory element(s) involved in nitrate-regulated NiR expression. While there were marked differences in the nitrate-induced GUS activity levels among transformants derived from the same construct, there were no dramatic changes in nitrate-induced GUS activity with progressive upstream deletions to −330 bp. It therefore is unlikely that there are important positive or negative regulatory elements between −3100 and −330 of the NiR promoter.

Table 1. Expression of NiR–GUS gene fusions in the leaf and root tissues of transgenic tobacco plants grown on ammonium- and nitrate-containing media

	Number of transformants		
Construct	Nitrate inducible	Constitutive	No activity
787 (−3100)	9/13	1/13	3/13
788 (−1730)	7/9	0/9	2/9
786-22 (−773)	7/8	0/8	1/8
786-B8 (−330)	7/9	1/9	1/9
786-6 (−200)	0/10	0/10	10/10

GUS activity was determined by histochemical localization as described in the Experimental procedures.

Table 2. GUS activity (nmol mg protein^{-1} h^{-1})[a] in the leaves and the roots of transgenic tobacco plants grown aseptically on ammonium- (A) or nitrate-containing (N) media

		Leaf		Root	
Construct	No.	A	N	A	N
787	1	33	58	–	50
(−3100)	6	–	124	–	215
	9	19	132	–	308
	11	–	116	174	1135
	12	37	233	35	607
788	1	–	166	184	825
(−1730)	6	87	634	51	324
	7	25	136	70	965
	9	56	275	18	832
786-22	3	–	151	36	949
(−773)	4	62	181	49	559
	6	–	109	–	229
	12	–	369	197	909
786-B8	1	–	167	32	521
(−330)	3	–	140	47	718
	4	–	173	20	393
	8	41	293	46	860
	10	38	33	60	41
786-6	8	–	–	–	–
(−200)	10	–	–	–	–
	13	–	–	–	–

Values presented are from one experiment. GUS activity assays were performed three times with similar results.

[a] Values presented are after the subtraction of background GUS activity observed in leaves (26 nmol mg protein^{-1} h^{-1}) and roots (51 nmol mg protein^{-1} h^{-1}) of untransformed tobacco plants.

−No GUS activity above background detected.

Effects of 5′ promoter deletions on tissue-specific expression

The tissue-specific pattern of NiR expression was investigated by localizing GUS activity histochemically in the sections of leaves, stems and roots of transgenic plants harboring the various NiR deletion constructs. Plants were grown on ammonium- and nitrate-containing media. It should be noted that for a given organ, variation in the staining intensity was observed among the independent transformed lines, which was consistent with the variation in GUS activity.

Leaf: most of the NiR 787 transformants (containing −3100 bp of the promoter region), grown on ammonium-containing medium, showed no detectable GUS activity in any leaf tissue, as indicated by the absence of blue color (Figure 2a), but when grown on nitrate-containing medium, they showed the presence of significant GUS activity (Figure 2b). The nitrate-induced GUS expression was localized mainly in the mesophyll tissue (both palisade and spongy parenchyma, Figure 2b) with little or no GUS activity present in other tissues such as the hairs, epidermis including stomata, and the midrib. GUS activity was, however, observed in minor veins running through the mesophyll tissues.

Progressive 5′ deletions of the NiR promoter to −330 bp did not affect the qualitative nitrate-induced tissue-specific expression as transgenic plants with truncated promoters exhibited a pattern of GUS expression similar to that described for the longest promoter fragment. In the absence of nitrate, the leaves of 786-B8 (Figure 2c) and those of 788 and 786-22 (data not shown) exhibited no GUS activity, while in its presence, GUS activity was observed in mesophyll tissue (Figure 2d). Further deletion of the promoter to −200 resulted in loss of nitrate inducibility of GUS expression, and no GUS activity was observed in any of the leaf tissues either in the absence (Figure 2e) or the presence of nitrate (Figure 2f).

A different pattern of GUS expression was observed in the leaves of a minority of the transformed lines (approximately 20%). Regardless of the size of the promoter (except −200), some lines showed basal GUS activity (in the absence of nitrate) that was present in the vascular and lower mesophyll (spongy parenchyma) tissues, but was absent in all other tissues including the trichomes, epidermis and stomata, and the midrib (data not shown). A similar pattern of tissue localization for GUS expression was noted for transformants in which GUS activity was not quantitatively affected by nitrate. In other lines, the addition of nitrate increased GUS activity in all the different tissues including the epidermis and trichomes.

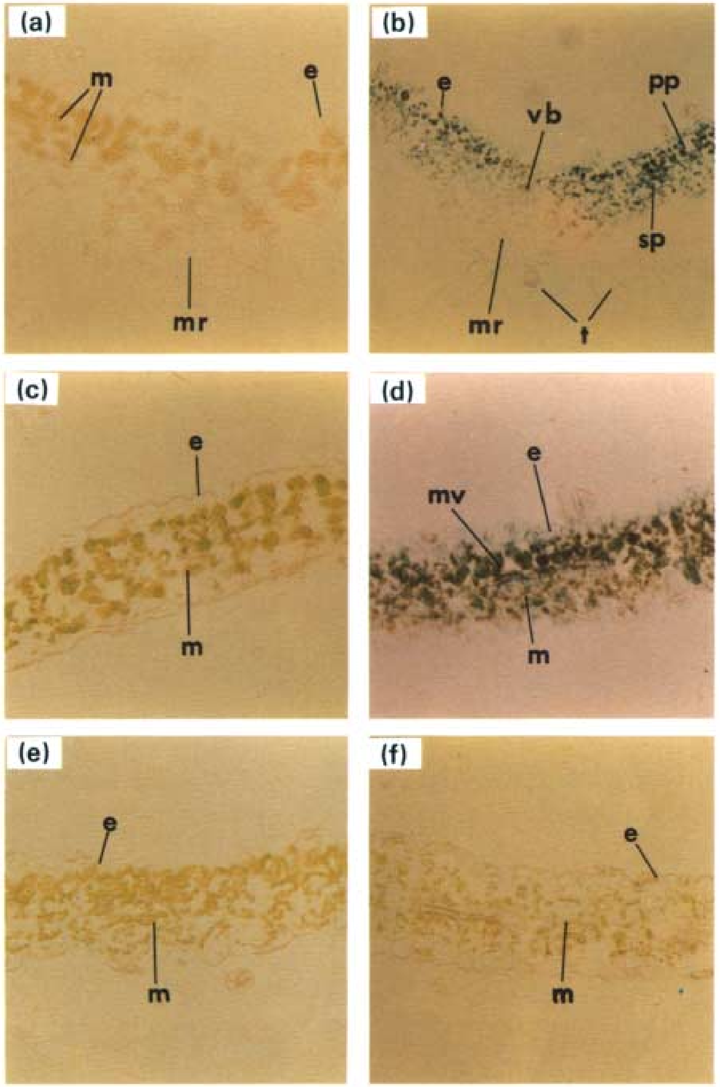
Stem: the expression of the various NiR–GUS constructs in the stems is shown in Figure 3. Transgenic plants carrying the 3.1 kb promoter fragment (787) grown in the presence of ammonium generally did not show GUS activity in any of the tissues (Figure 3a). However, when plants were grown on nitrate-supplemented medium, GUS activity was present in the stems (Figure 3b). The GUS gene was expressed in various stem tissues, but the expression was most pronounced in the vascular tissues (Figure 3b).

The 788, 786-22 and 786-B8 plants also showed little GUS activity in the stems in the absence of nitrate (Figure 3c; shown for 786-B8 stem), but in the presence of nitrate expression of the GUS gene was observed (Figure 3d). The pattern of expression in the various tissues was similar to that observed in the stems of plants transformed with the largest promoter construct (787). In some of the transgenic lines, faint GUS activity was inconsistently observed in the xylem even in the absence of nitrate (data not shown). No GUS activity was detected in the stems of 786-6 (−200) plants either in the absence (Figure 3e) or the presence (Figure 3f) of nitrate.

Root: Figure 4 shows the pattern of NiR–GUS expression in the roots of transgenic plants harboring the various constructs in the absence and the presence of nitrate. In the absence of nitrate, the majority of the transformants of 787 (Figure 4a), 788 and 786-22 (data not shown) and 786-B8 (Figure 4d) showed little or no detectable GUS activity throughout the length of the root including the root hair and the root tip. In the presence of nitrate, however, GUS expression was induced, and was mostly localized in the vascular cylinder and the adjacent cortical cells (Figure 4b and e). Nitrate-induced GUS activity was absent in the epidermis and the root tip (Figure 4c) in all cases. In some transformants weak GUS expression was detected in the vascular tissues and cortex even in the absence of nitrate (data not shown). None of the plants with the NiR promoter deleted to −200 position showed any detectable GUS activity in the absence (Figure 4f) or the presence of nitrate (Figure 4g).

Induction experiments

Experiments were performed to study the kinetics of NiR–GUS induction following nitrate treatment, and to determine in which organs the NiR gene is induced first. To this end, eight 12-week-old seedlings grown on ammonium-containing medium, were used. Seedlings were transferred to a liquid nutrient medium and after 16–20 h potassium nitrate was added to a final concentration of 20 mM. Individual seedlings were harvested at times 0, 5, 15 and 30 min, and 1, 2, 6 and 24 h after the addition of nitrate and stained for GUS activity. The results of such an experiment with a 787 transformed line are shown in Figure 5. No detectable GUS activity was induced in either the roots or the leaves 1 h after nitrate treatment. In seedlings treated for 2 h, however, weak GUS activity was present in the roots with a significant increase between 2



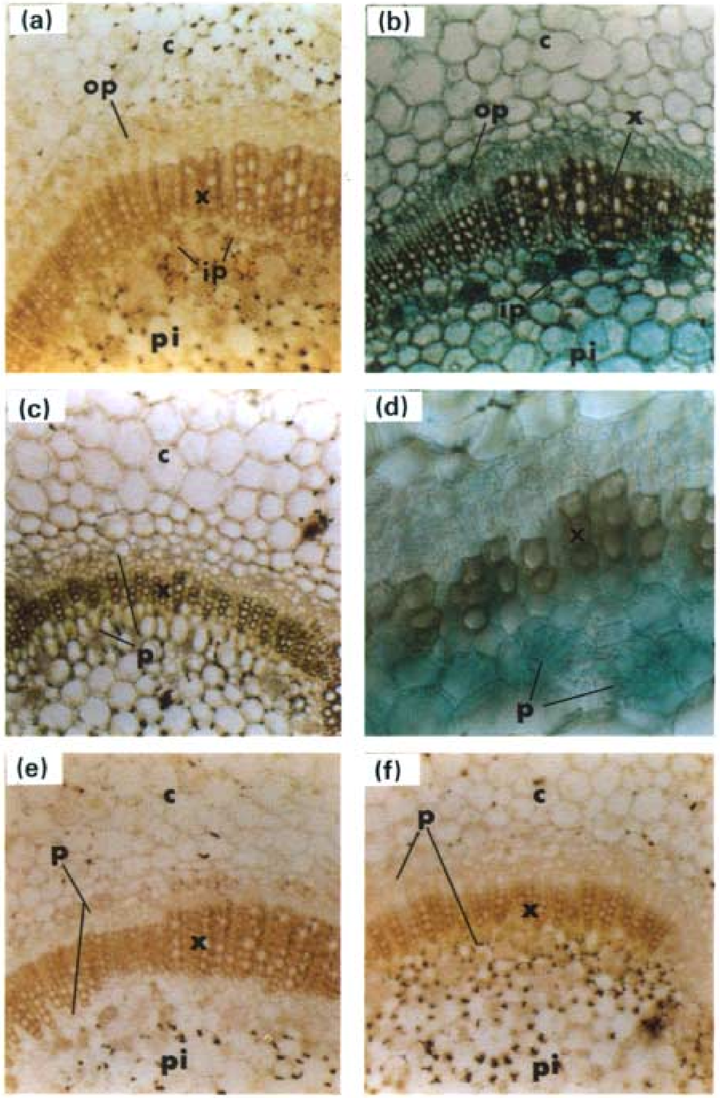
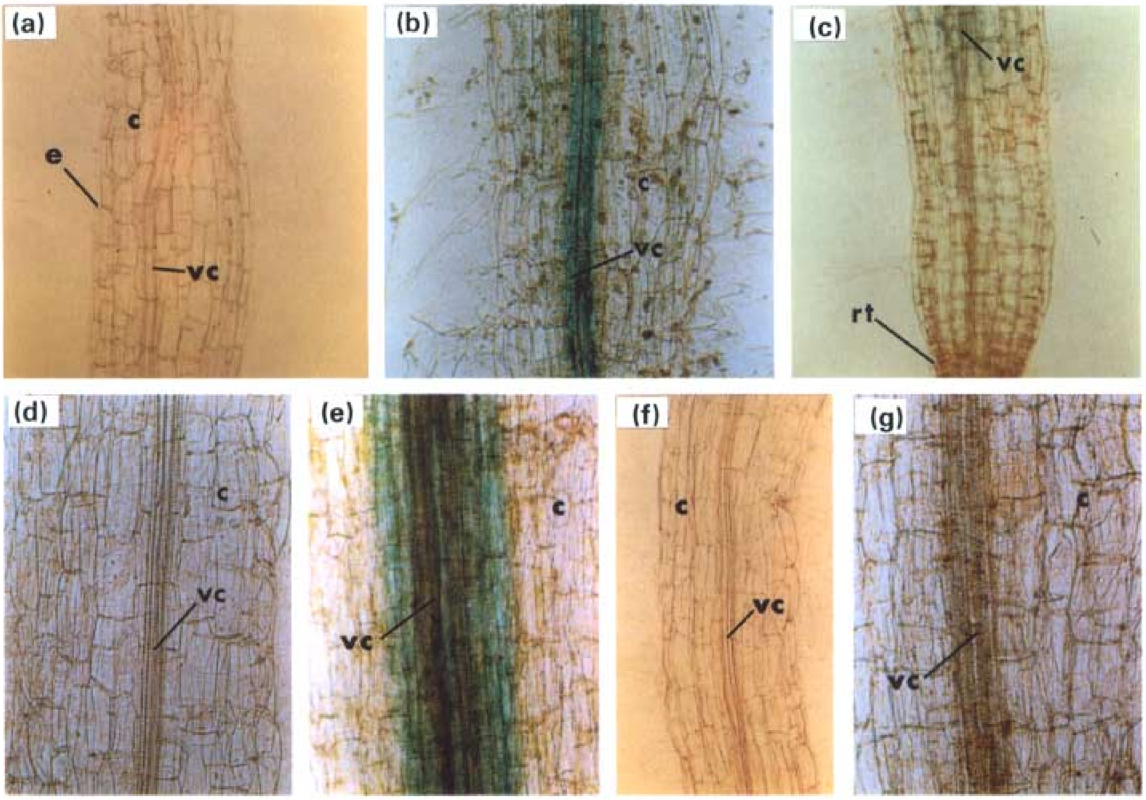


Figure 2 (left). Histochemical localization of GUS activity in the leaves of transgenic tobacco plants harboring the various NiR–GUS constructs.
Leaves were stained and sectioned as described in Experimental procedures (all × 96).
Transverse sections through leaf from: (a) NiR 787 plant grown in the presence of ammonium. (b) NiR 787 plant grown in the presence of nitrate. (c) NiR 786-B8 plant grown in the presence of ammonium. (d) NiR 786-B8 plant grown in the presence of nitrate. (e) NiR 786-6 plant grown in the presence of ammonium. (f) NiR 786-6 plant grown in the presence of nitrate.
Abbreviations: e, epidermis; m, mesophyll; mr, midrib; mv, minor vein; pp, palisade parenchyma; sp, spongy parenchyma; t, trichome; vb, vascular bundle.

Figure 3 (right). Histochemical localization of GUS activity in the stems of transgenic tobacco plants harboring the various NiR–GUS constructs.
Stems were sectioned and stained as described in Experimental procedures.
Cross-sections through stem from: (a) NiR 787 plant grown in the presence of ammonium (×52). (b) NiR 787 plant grown in the presence of nitrate (×65). (c) NiR 786-B8 plant grown in the presence of ammonium (×52). (d) NiR 786-B8 plant grown in the presence of nitrate (×110). (e) NiR 786-6 plant grown in the presence of ammonium (×52). (f) NiR 786-6 plant grown in the presence of nitrate (×60).
Abbreviations: c, cortex; ip, inner phloem; op, outer phloem; p, phloem; pi, pith; x, xylem.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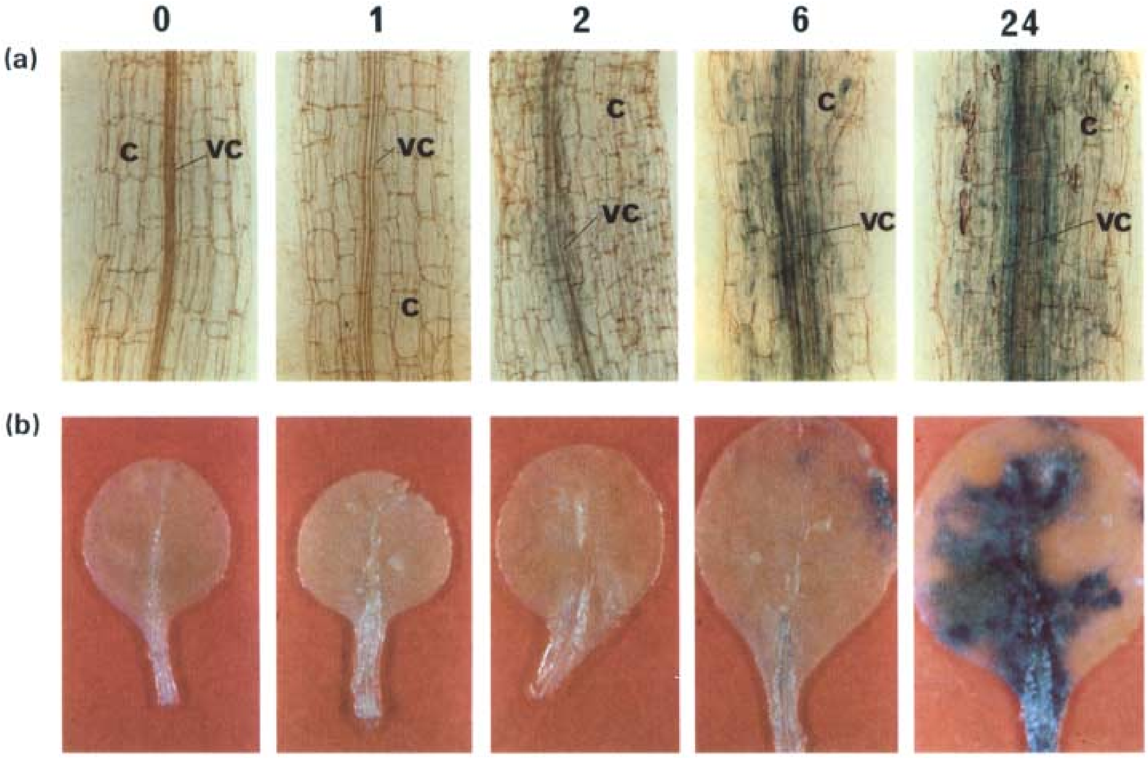
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and 6 h and thereafter (Figure 5a). GUS activity in the roots was localized mainly in the vascular cylinder and the cortex (Figure 5a), which is the same as that seen for roots of plants grown continually in the presence of nitrate (Figure 4). In the leaves, a significant induction of GUS activity occurred 24 h after treatment with nitrate although some GUS activity was detectable after 6 h (Figure 5b). It should be noted that while the timing of appearance of GUS activity and the increase in the intensity of GUS staining over time is an accurate reflection of the induction of NiR–GUS by nitrate, the GUS staining in the leaves was not uniform and reflects differential uptake of the histochemical substrate. The appearance of nitrate-induced GUS activity in the root, thus, precedes that in the leaf. Experiments were also performed with 788, 786-22 and 786-B8 plants with similar results.

Discussion

In higher plants, the expression of the NR and NiR genes is primarily regulated by the presence of nitrate (Back *et al.*, 1988; Cheng *et al.*, 1986; Crawford *et al.*, 1986; Lahners *et al.*, 1988). However, there is considerable evidence that their expression is also regulated by other stimuli such as light (Bowsher *et al.*, 1991; Deng *et al.*, 1990; Galangau *et al.*, 1988), sucrose (Cheng *et al.*, 1992), nitrogen metabolites (Deng *et al.*, 1991), and hormones (Lu *et al.*, 1990, 1992). The leaves and/or the roots are the major sites of nitrate assimilation depending upon the plant type, although nitrate reduction has also been shown to take place in stems, embryos and other plant parts (Andrews, 1986; Guerrero *et al.*, 1981; Salisbury and Ross, 1985). While a variety of organs are able to assimilate nitrate, the precise localization of the expression of the assimilatory enzymes to specific cell types is not entirely clear. NiR is a plastid localized enzyme (see Guerrero *et al.*, 1981 for review), and based upon the biochemical evidence both NR and NiR appear to be expressed in the mesophyll tissues of the leaf. There is, however, relatively little known of their tissue localization in other plant organs.

In earlier work, a 3.1 kb 5′ upstream region of the spinach NiR gene promoter was shown to regulate nitrate-induced expression of the GUS reporter gene (Back *et al.*, 1991). In the present report, results of experiments aimed at delineating the region(s) involved in nitrate-regulated expression of NiR are described. Various deletion constructs of the NiR promoter, fused to the GUS reporter gene, were transformed into tobacco, and the expression of the GUS reporter gene in different tissues and its regulation by nitrate were analyzed in a large number of transgenic lines. Transgenic plants with NiR promoter ranging from −3100 to −330 upstream of the transcription initiation site, when grown in the presence of nitrate, showed a large increase in GUS activity in all the different organs analyzed. There was considerable variability with respect to GUS activity among the different lines generated using the same construct. These variations were in the levels of GUS activity in the presence of nitrate as well as in the levels of constitutive GUS activity in the absence of nitrate. In two of the lines no change in the level of GUS activity was seen whether or not nitrate was present. This variability in GUS expression among transgenic lines carrying the same construct is almost certainly due to the different chromosomal location of the transgene. When the various deletion constructs are compared, there are no obvious differences among them with regard to the levels of GUS activity in the presence or absence of nitrate. Therefore, it is unlikely that any important regulatory elements involved in nitrate regulation of NiR expression are present in the region of the NiR promoter between −3100 and −330.

Since the −330 region of the spinach NiR promoter appears to contain all the *cis*-acting elements required for nitrate regulation of NiR expression, its DNA sequence was compared with the 5′ flanking sequences for the NR genes of tomato (Daniel-Vedele *et al.*, 1989), tobacco (Vaucheret *et al.*, 1989), barley (Miyazaki *et al.*, 1991), spinach (Prosser, personal communication) and *Arabidopsis* (Wilkinson and Crawford, 1991). Such comparisons failed to reveal any conserved sequence that is present in all of these promoters. There are two possible reasons for this result: first, the factor(s) mediating nitrate induction of NiR and NR genes can possibly bind to diverse binding sites sharing a small core sequence similar to that reported

Figure 4 (top). Histochemical localization of GUS activity in the roots of transgenic tobacco plants harboring the various NiR–GUS constructs.
Roots were stained and mounted as described in Experimental procedures.
Squashes of root from: (a) NiR 787 plant grown in the presence of ammonium (×67). (b) NiR 787 plant grown in the presence of nitrate (×90). (c) NiR 787 plant grown in the presence of nitrate showing absence of GUS activity in the root tip (×90). (d) NiR 786-B8 plant grown in the presence of ammonium (×80). (e) NiR 786-B8 plant grown in the presence of nitrate (×67). (f) NiR 786-6 plant grown in the presence of ammonium (×67). (g) NiR 786-6 plant grown in the presence of nitrate (×67).
Abbreviations: c, cortex; e, epidermis; rt, root tip; vc, vascular cylinder.

Figure 5 (bottom). Time course of induction of the NiR–GUS gene fusion by nitrate in the roots (a) (all × 87) and the leaves (b) (all × 2) of a NiR 787 transformant.
Times (in hours) at which the plants were sampled following nitrate treatment are shown at the top. Induction experiments and GUS activity localization were performed as described in Experimental procedures.
Abbreviations: c, cortex; vc, vascular cylinder.

for the G-box binding factors (Schindler *et al.*, 1992), and such sequences could be missed by DNA sequence comparisons. Alternatively, they bind to completely different sites in a fashion analogous to that described for the CCAAT/enhancer-binding protein (Lamb and McKnight, 1991).

A further deletion in the NiR promoter to −200 results in a total loss of expression of the GUS reporter gene. There are two possible explanations for this result. One is that the deletion to −200 position removes all or part of the element involved in regulation of NiR expression by nitrate, and in the absence of this element there is no expression. It should be noted that in the absence of nitrate, there was also no GUS expression in some of the 787 plants (carrying the longest promoter fragment). An alternative explanation as to why the −200 bp construct shows no GUS expression could be that another promoter element that is absolutely required for any expression might have been deleted, with the nitrate response element being located closer to the transcription start site.

The induction of GUS activity in the NiR–GUS plants upon the addition of nitrate was fairly similar to that seen for the induction of tobacco NR activity by nitrate (Galangau *et al.*, 1988), and was also consistent with the induction of NiR protein in maize roots and shoots (Kramer *et al.*, 1989). Significant GUS activity appeared first in roots approximately 2 h after the addition of nitrate followed by its appearance in leaf petioles between 2 and 6 h, and in the leaf blade and the minor veins between 6 and 24 h. In roots, GUS activity was localized in the vascular cylinder and the surrounding cortical cells which was similar to the situation in the roots of plants grown constantly in the presence of nitrate.

The NiR–GUS gene fusion was expressed in all three organs investigated, i.e. leaf, stem and root, and within these organs NiR-regulated GUS activity was localized to certain cell types. In leaves, GUS activity was primarily located in the mesophyll tissue comprising palisade and spongy parenchyma, and the embedded minor veins with little or no expression in the epidermis and the midrib. In stems, GUS staining was most pronounced in the vascular tissues, particularly phloem, and to a lesser extent in xylem and pith tissues. In roots, GUS activity was predominantly localized in the vascular cylinder and adjacent cortical cells, but was absent in the epidermis. Furthermore, there was no expression in the root tip, a result similar to that seen for maize NR (Long *et al.*, 1992). Progressive 5′ deletions in the promoter to −330 did not alter the qualitative, tissue-specific pattern of NiR expression. This supports the notion that there are no regulatory sequences in the −3100 to −330 region of the NiR promoter important for its tissue-specific expression.

In summary, the region of the spinach NiR promoter between −330 and the transcription initiation site contains all sequences required for nitrate inducibility and tissue-specific expression of NiR. There are several possible factors that hypothetically could control this tissue specificity. First, the accessibility of nitrate to different tissues could be limiting, although this seems unlikely for the root epidermal tissue. Second, the regulatory protein(s) required for increased expression of NiR in the presence of nitrate might only be produced in certain cells, for example, those cells containing abundant plastids. Finally, there could be other *cis*-acting elements regulating the tissue-specific expression, with the nitrate response element being absolutely required for any transcription to occur. The expression of NR and NiR are coordinately regulated by nitrate and given the cytotoxic nature of nitrite, a product of nitrate reduction, one would expect NiR to be expressed in any cell expressing NR. Therefore, it will be of interest to determine if the pattern of NR expression in different tissues is similar to that observed for the spinach NiR promoter.

Experimental procedures

Generation of 5′ deletions in the NiR promoter, construction of the NiR–GUS gene fusions and plant transformations

The clone NiR 792 containing the NiR promoter sequences is described in Back *et al.* (1991) and Figure 1, and was used for the generation of the 5′ end deletions. The deletion −1730 (NiR 788) was a *Xba*I–*Bgl*II fragment of NiR 792 while other deletions (−773, −330 and −200) were produced by Exonuclease III/Mungbean nuclease treatment of NiR 792 following digestion with *Kpn*I and *Xba*I. After the Exonuclease III/Mungbean nuclease digestion, the plasmids were religated to *Eco*RI linkers, digested with *Eco*RI and the ends polished with Klenow. *Sal*I linkers were then added and the truncated NiR promoters excised as *Sal*I–*Bgl*II fragments were ligated between the *Sal*I and *Bam*HI sites of the plant transformation vector pBI101 (Jefferson *et al.*, 1987) to position them properly with reference to the GUS gene. The *Sal*I–*Bgl*II (−3100) and the *Xba*I–*Bgl*II fragments of NiR 792 were also inserted into pBI101 to give plasmids NiR 787 (−3100) and NiR 788 (−1730). *Nicotiana tabacum* leaf transformations were performed as in Rothstein *et al.* (1987). Nine to 13 primary transformants (each derived from an independently transformed leaf disc) for each construct were selfed and F_1 seed collected. Plants obtained from F_1 seed were used for further analyses.

Plant cultivation

In our earlier work where the plants were grown in a soil/perlite mixture, we observed a considerable level of GUS activity before the additon of nitrate (Back *et al.*, 1991), most likely due to the presence of traces of nitrate in the soil mixture produced as a result of microbial ammonium oxidation. This prevented us from making a precise estimation of constitutive GUS activity and the degree of nitrate induction in various transformed lines. Since the NiR–GUS fusion appears to be sensitive even to low nitrate levels, we decided to grow plants under sterile conditions in the absence and the presence of nitrate. In all cases, plants were grown in an incubator maintained at 25°C with a 16 h light/8 h dark cycle. Seeds were germinated aseptically on solid media (0.9% agarose) containing either ammonium (NH_4–S salts of Evola (1983), pH 5.8) or ammonium and nitrate (Murashige and Skoog's salts, pH 5.8) and 50 $\mu g\ ml^{-1}$ kanamycin. The NH_4–S medium is a modified Murashige and Skoog's medium in which KNO_3 and NH_4NO_3 have been replaced with 25 mM (NH_4Cl + succinic acid) and 12.7 mM KCl; with NH_4Cl as the sole nitrogen source (Evola, 1983). After 8–10 weeks, seedlings resistant to kanamycin were used for experimental work or transferred to the same media in GA–7 containers.

Spectrometric GUS assays

GUS activity was measured by the spectrophotometric assay utilizing *p*-nitrophenyl glucuronide as substrate (Jefferson, 1987). GUS assays were performed three times. Enzyme activity is expressed as nmol mg $protein^{-1}\ h^{-1}$.

Histochemical staining of GUS activity

For the initial screening of transformants for induction of GUS activity by nitrate, leaf and root pieces were cut from plants grown on ammonium- and nitrate-containing media and stained for GUS activity as described in Jefferson *et al.* (1987). Tissues were submerged in GUS reaction mixture containing 1 mM X-Gluc, briefly vacuum-infiltrated and incubated at 37°C for 16 h to 2 days. After the development of the blue color, tissue was washed several times in 50 mM phosphate buffer (pH 7.0) and observation made.

Sectioning and microscopy

Pieces of stem, leaf and root were harvested from control (grown in the presence of ammonium) and nitrate-induced (grown in the presence of nitrate) plants. For stems, free-hand sections were cut and stained as above. Leaves and roots were stained prior to sectioning and squash preparation, respectively. For transverse sections of the leaf, pieces of leaf tissue were placed in O.C.T. (Tissue-Tek II embedding medium), frozen in liquid nitrogen and cut to 10–12 µm thick sections using a IEC CTF microtome-cryostat maintained at −20°C. Frozen sections were transferred to slides. Leaf and stem sections, and root squashes were all mounted in glycerol, and examined and photographed using a Zeiss-Jena Jenalumar Contrast microscope.

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Induction experiments

Induction experiments were done as follows. Eight to 10 kanamycin-resistant seedlings grown on NH_4–S medium were transferred to modified liquid NH_4–S medium without ammonium, with the seedlings supported on nylon screen. They were then allowed to recover for 16 h. Following recovery, potassium nitrate was added to 20 mM final concentration to induce the NiR–GUS gene fusion. Entire individual seedlings were removed at times 0, 5, 15 and 30 min, and 1, 2, 6 and 24 h after the addition of nitrate, washed with water and incubated at 37°C in GUS activity localization buffer for 24–36 h. Following incubation the seedlings were cleared in ethanol, washed, and observations made on GUS activity localization. When entire seedlings were stained for activity localization, an uneven distribution of GUS activity was observed in the leaves (Figure 5b), and this was almost certainly due to an uneven uptake of GUS substrate since leaves cut into small peices stained fairly uniformly.

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