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TECHNICAL ADVANCE

NAN fusions: a synthetic sialidase reporter gene as a sensitive and versatile partner for GUS

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Summary

GUS continues to be the reporter of choice for many gene fusion applications, due to the unparalleled sensitivity of the encoded enzyme and the ease with which it can be quantified in cell-free extracts and visualized histochemically in cells and tissues. A compatible and functionally equivalent reporter gene would facilitate dual promoter studies and internal standardization of expression analyses in the same plant. A search for a candidate enzyme activity not found in plants, which might form the basis of a novel GUS-compatible reporter system, led us to investigate *nan*H, a *Clostridium perfringens* gene which encodes the so-called 'small' cytoplasmic sialidase. Expression of the native, AT-rich *nan*H gene in transgenic plants did not, however, result in detectable sialidase activity. For this reason, a codon-optimized derivative, *NAN*, was synthesized which possesses a GC content similar to that found in highly expressed plant genes. NAN enzyme activity was expressed at high levels in both stably and transiently transformed cells, possessed kinetic and stability properties similar to those of GUS, and showed optimal activity in GUS buffer. Moreover, NAN and GUS activity could be visualized simultaneously in polyacrylamide gels using the corresponding methylumbelliferone-based substrates, and in whole seedlings and tissue sections using the histochemical substrates 5-bromo-4-chloro-3-indolyl α-D-*N*-acetylneuraminic acid (X-NeuNAc) and 5-bromo-6-chloro-3-indolyl β-D-glucuronide (X-GlucM), respectively.

Keywords: synthetic gene, sialidase, NAN, reporter genes, transgenic plants, GUS.

Introduction

The introduction of the GUS reporter gene (Jefferson *et al.*, 1987) revolutionized the analysis of plant gene expression. Its success can be attributed to several factors: the absence of an endogenous GUS activity in plants; the stability and high specific activity of the GUS enzyme which confers unparalleled sensitivity; the tolerance of the GUS enzyme to both N- and C-terminal fusions; the availability of sensitive, technically simple, inexpensive and rapid assays for GUS activity; and, crucially, the availability of histochemical substrates that permit visualization of GUS activity in plant cells and tissues. For these reasons, GUS continues to be the reporter gene of choice for the analysis and identification of genes that are expressed in a tissue-specific or developmentally regulated manner.

The utility of the GUS gene could, however, be extended if a complementary reporter system were developed which encoded an enzyme with similar kinetic properties that could be assayed as easily as GUS, using a similar range of substrates. This might also, in theory, enable both reporter enzymes to be assayed or visualized simultaneously in the same tissue sample. Such a GUS-compatible reporter gene system would facilitate additional gene fusion-based analyses in transgenic plants already containing the GUS gene, and provide a convenient internal standard for use in transient expression analyses and other investigations.

Various reporter genes have been developed for use in plants, including those encoding neomycin phosphotransferase (NPTII) (Bevan *et al.*, 1983); chloramphenicol acetyltransferase (CAT) (Herrera-Estrella *et al.*, 1983); β-galactosidase (β-GAL) (Teeri *et al.*, 1989); luciferase (LUC) (Ow *et al.*, 1986); and, more recently, the jellyfish green

fluorescent protein (GFP) (Chalfie, 1995) and the red fluorescent protein from *Discosoma* sp. (dsRED) (Jach *et al.*, 2001). However, all possess one or more disadvantages relative to GUS. There may, for example, be problems with high endogenous background activity (β-GAL), low sensitivity (GFP, dsRED), a technically demanding (LUC, GFP, dsRED) or time-consuming assay (CAT, NPTII), or it may be difficult (β-GAL, LUC) or not possible (NPTII, CAT) to localize reporter gene activity in cells and tissues. Thus, for technical and operational reasons, none of the reporter genes currently available is ideal for use in combination with GUS.

We initiated a search for a novel, GUS-compatible reporter gene system by investigating the ability of cell-free extracts of tobacco (*Nicotiana tabacum*) to cleave a range of candidate commercially available colorimetric, fluorogenic and histochemical substrates. This study revealed that tobacco protein extracts were unable to cleave the sialidase substrates 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUN), 2-*o*-(*p*-nitrophenyl)-α-D-*N*-acetylneuraminic acid (pNP-N) and 5-bromo-4-chloro-3-indolyl α-D-*N*-acetylneuraminic acid (X-NeuNAc). A more extensive investigation confirmed that, in addition to tobacco, an endogenous sialidase activity was also absent in cell-free extracts of *Arabidopsis*, rice, tomato and soybean.

Sialidases (syn. neuraminidases, EC 3.2.1.18) catalyse the cleavage of terminal *N*-acylneuraminate (sialic acid) residues from glycoproteins, glycolipids and polysaccharides. They are found in most animal lineages, from Echinodermata through Mammalia, and also in diverse microorganisms many of which are animal commensals or pathogens. In mammalian cells, sialidases are found in the cytoplasm, in lysosomes, and associated with the plasma membrane (Miyagi *et al.*, 1992), but the specific activity of these enzymes is typically one to two orders of magnitude lower than that of the microbial enzymes. The bacterial sialidases, most of which are either surface-bound or secreted enzymes, are thought to play a role in microbial nutrition and, more controversially, in pathogenesis (Corfield, 1992). Sialidases and their sialyl substrates have not been reported from plants (Corfield and Schauer, 1982).

The lack of an endogenous sialidase activity in plants and the commercial availability of sensitive sialidase substrates suggested that a gene encoding a high-specific-activity bacterial sialidase might be developed as a GUS-compatible reporter gene. We describe the total synthesis of a codon-optimized derivative of the *nan*H gene which encodes the 'small' cytoplasmic sialidase of *Clostridium perfringens* (Roggentin *et al.*, 1988; Kruse *et al.*, 1996). We show that the resulting synthetic gene, designated *NAN*, is efficiently expressed in plant cells, and demonstrate its use as a highly sensitive reporter gene in its own right and as an ideal partner for use with GUS.

Results and discussion

Synthesis of NAN, a sequence-modified nanH derivative, for optimal expression in plants

Our attempts to express a PCR-amplified copy of the native *nan*H gene of *C. perfringens* (Roggentin et al., 1988) in plant cells were not successful, despite the fact that the encoded sialidase activity was expressed at a high level in *Escherichia coli* (data not shown). We concluded that the lack of expression in plants was probably due to the low GC content of *nan*H (31.8%) and, in particular, the frequent occurrence of AT-rich sequence elements that are either known or predicted to act as cryptic polyadenylation signals in plant cells (Rothnie *et al.*, 1994). We therefore decided to construct a synthetic gene, designated *NAN*, based on the amino acid sequence of the NANH sialidase, with the aim of minimizing the potential for deleterious post-transcriptional processing events while optimizing codon usage, mRNA stability and translational efficiency (Koziel *et al.*, 1996).

In designing the synthetic *NAN* sequence, the following criteria were used: (i) codons were selected based on the average codon usage frequencies found in nuclear genes of *Arabidopsis thaliana* and *N. tabacum*; (ii) the overall GC content of the coding sequence was increased from 31.8% (native *nan*H) to 43.8%, a GC content similar to that found in highly expressed plant genes (Murray *et al.*, 1989; Koziel *et al.*, 1996); (iii) potential polyadenylation signal sequences, e.g. AATAAA, or similar hexamers with a one base mismatch which occur frequently throughout the native *nan*H open reading frame (ORF), and the sequence TTTGTA, were eliminated (Rothnie *et al.*, 1994); (iv) ATTTA strings, which are associated with mRNA instability (Perlak *et al.*, 1991), were also avoided; (v) no sequences with the potential to form significant mRNA stem-loops (stem length = 10 bp; = 22 bonds) were included; (vi) potential cryptic intron splice sites were avoided; and (vii) to ensure optimal initiation of translation, the Kozac consensus sequence AACA, found at positions −1 to −4 relative to the initiating ATG in many plant genes (Joshi, 1987; Kozak, 1999), was included in the synthetic *NAN* gene.

Of the three gene construction methods used (see Experimental procedures), the PCR synthesis method of Stemmer *et al.* (1995) was the most effective, based on the error rate, expense and time involved. The sequence of the synthetic *NAN* gene is shown in Figure 1.

NAN activity can be extracted and assayed in GUS buffer

NAN activity was determined under different assay conditions using sonicated extracts of *E. coli* harbouring pROK219-NAN (see Figure 3 below) as the enzyme source and MUN as the substrate. The pH profile of NAN

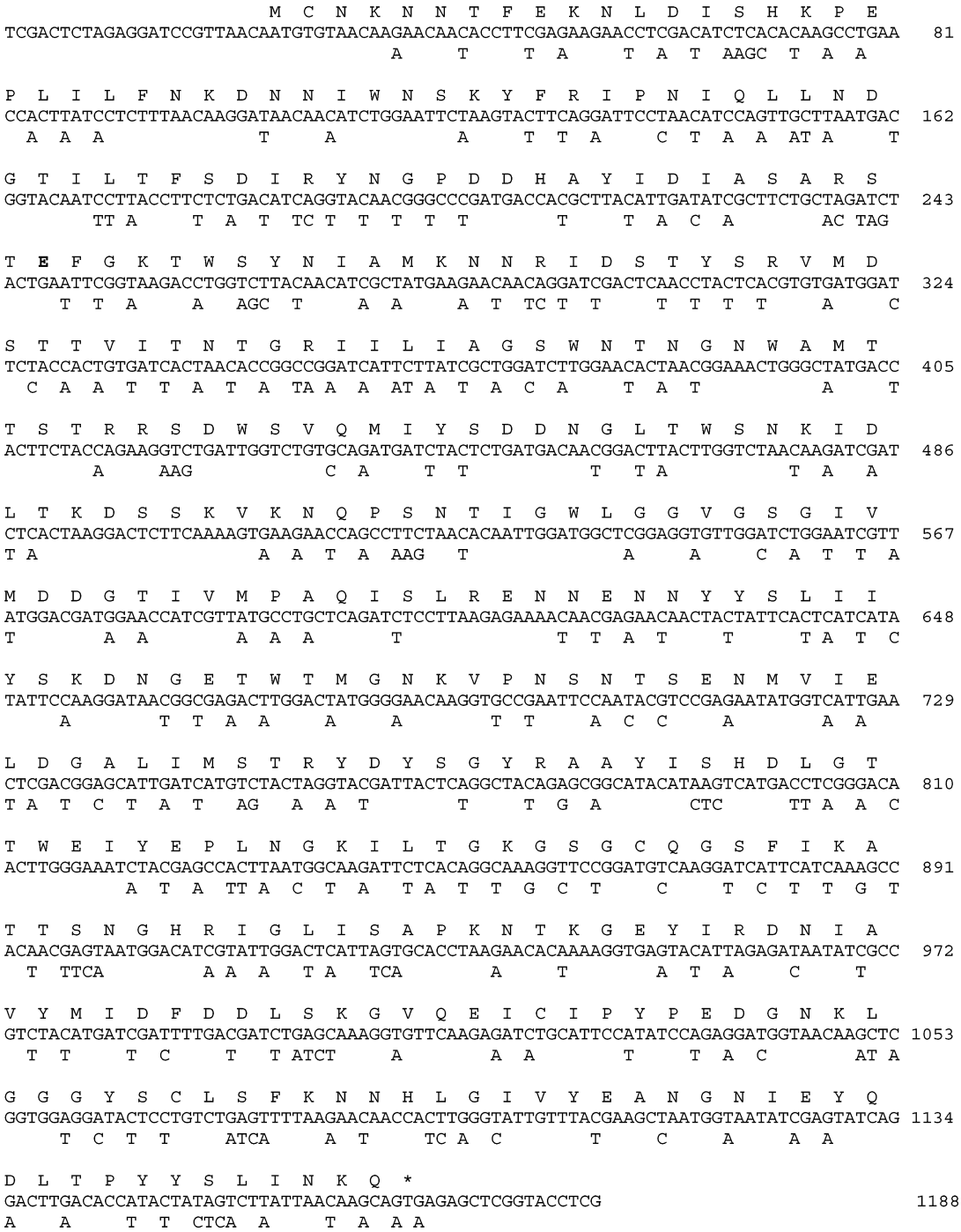

Figure 1. Nucleotide sequence of the synthetic sialidase gene, *NAN*.
Numbered lines show the nucleotide sequence of the *NAN* gene; lines underneath show nucleotides of the native *nanH* gene of *Clostridium perfringens* (GenBank Accession No. Y00963) that were replaced in *NAN*. The deduced amino acid sequence is shown above the *NAN* nucleotide sequence; the D75E substitution is highlighted in bold.

activity was measured in a range of 50 mM sodium citrate buffers, pH 4.0–8.5, and compared with the activity of the native NANH enzyme (Figure 2). NAN was active over a wide range of pH values, with optimal activity at around pH 6.5–7.0, which represents a slight shift towards alkaline conditions relative to NANH. This may be due to the accidental introduction of a codon substitution mutation into the *NAN* ORF which replaces a highly conserved aspartic acid residue at position 75 with glutamic acid (D75E) (Figure 1).

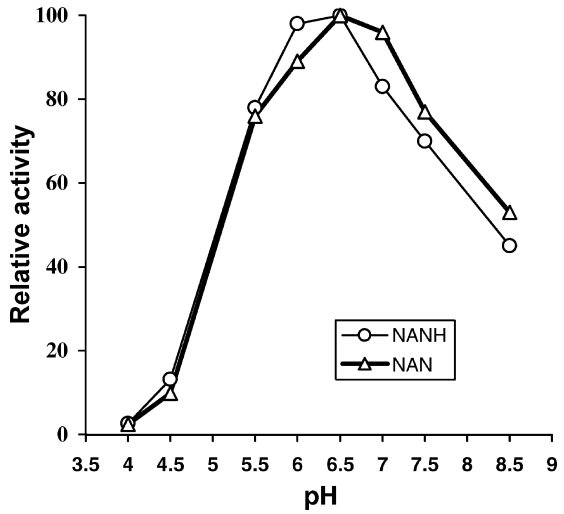

Figure 2. A comparison of the pH–activity profiles of NAN and NANH. NAN and NANH activities were measured in a range of 50 mM sodium citrate buffers differing with respect to pH, using MUN as substrate. Relative activity of each enzyme is shown as a function of pH.

Comparative assays of NAN activity in different buffer systems (sodium acetate, sodium phosphate and sodium citrate), and at various ionic strengths (50, 100 and 200 mM), indicated that NAN, like GUS, shows optimal activity in a 50 mM sodium phosphate buffer at pH 7.0 (data not shown). The combined effects of incorporating the additional components used in the standard GUS buffer were also investigated: 1 mM EDTA, 0.1% Triton X-100, and 10 mM β-mercaptoethanol (Jefferson *et al.*, 1987). The results showed that NAN could be extracted and assayed in GUS buffer with no significant compromise of its performance (data not shown).

NAN and GUS possess similar kinetic properties

In order to compare the kinetic properties of the encoded enzymes, the *NAN* and GUS genes were cloned into the T7 promoter-controlled vectors pET3a and pET24d, respectively, and expressed at high level in *E. coli* (data not shown). Total protein extracts containing equivalent amounts of each enzyme, based on band-intensity comparisons in Coomassie blue-stained polyacrylamide gels, were used to determine V_{max} and K_m values for NAN and GUS for the substrates MUN and 4-methylumbelliferyl β-D-glucuronide (MUG), respectively. NAN showed a V_{max} value of 3370 μmol min^{-1} mg^{-1} enzyme, while the value for GUS was 998 μmol min^{-1} mg^{-1} enzyme. The corresponding K_m values for MUN and MUG were 0.20 and 0.17 mM, respectively. Thus, while NAN and GUS showed similar affinities for their substrates, the specific activity of NAN was approximately 3.4-fold greater than that of GUS.

NAN expression in stably transformed tobacco plants

Nicotiana tabacum var. Samsun was stably transformed with derivatives of the vector pBIB-HYG (Becker, 1990), which contained the *NAN* gene under transcriptional control of the CaMV35S promoter (CaMV-NAN plants) or the promoter of a Rubisco small subunit (*rbcS*) gene (Mazur and Chui, 1985; rbcS-NAN plants) (Figure 3). NAN specific activity was determined in cell-free extracts of eight primary transformant plants and their seedling progeny using MUN as the substrate. CaMV-NAN and rbcS-NAN plants contained average NAN activities of 200 (±87) and 368 (±163) nmol MU min^{-1} mg^{-1} protein, respectively, in leaf tissue.

A comparison of the NAN activity levels observed in leaves of CaMV-NAN plants with the highest reported levels of GUS activity found in leaves of CaMV-GUS plants (64–119 nmol MU min^{-1} mg^{-1} protein; Benfey *et al.*, 1989), indicates a more than twofold higher level of NAN expression (relative to GUS) from the same promoter. This is consistent with the more than threefold difference in specific activity observed for the *E. coli*-expressed NAN and GUS enzymes (as discussed above), and suggests that the sensitivity of NAN as a reporter enzyme in plants is at least equal to, and probably exceeds, that of GUS. Transgenic lines expressing high levels of NAN activity showed no abnormalities in development, growth, fertility or germination.

NAN and GUS show similar half-lives in vivo *and* in vitro

In order to provide an estimate of the *in vivo* half-life of NAN, we took advantage of the fact that in rbcS-NAN plants, NAN expression was directed by the light-regulated *rbcS* promoter which is rapidly downregulated when plants are transferred to continuous darkness (Giuliano *et al.*, 1988). On the assumption that this would prevent further synthesis of NAN, an rbcS-NAN plant was placed in total darkness and the declining level of NAN activity was monitored in leaves over several days (data not shown). The *in vivo* half-life determined in this way was ≈60 h. The *in vitro* half-life of NAN activity in total protein extracts of CaMV-NAN plants prepared in GUS buffer was found to be at least 1 month for extracts stored at 4, −20 or −70°C, and ≈60 h at room temperature. These data indicate that the stability of NAN, both *in vivo* and *in vitro*, is very similar to that reported for GUS (Jefferson *et al.*, 1987).

Histochemical localization of NAN and GUS activity in stably transformed plants

NAN activity was localized in transgenic plants using the histochemical substrate X-NeuNAc. Localization of NAN activity in whole seedlings of CaMV-NANr, a CaMV-NAN

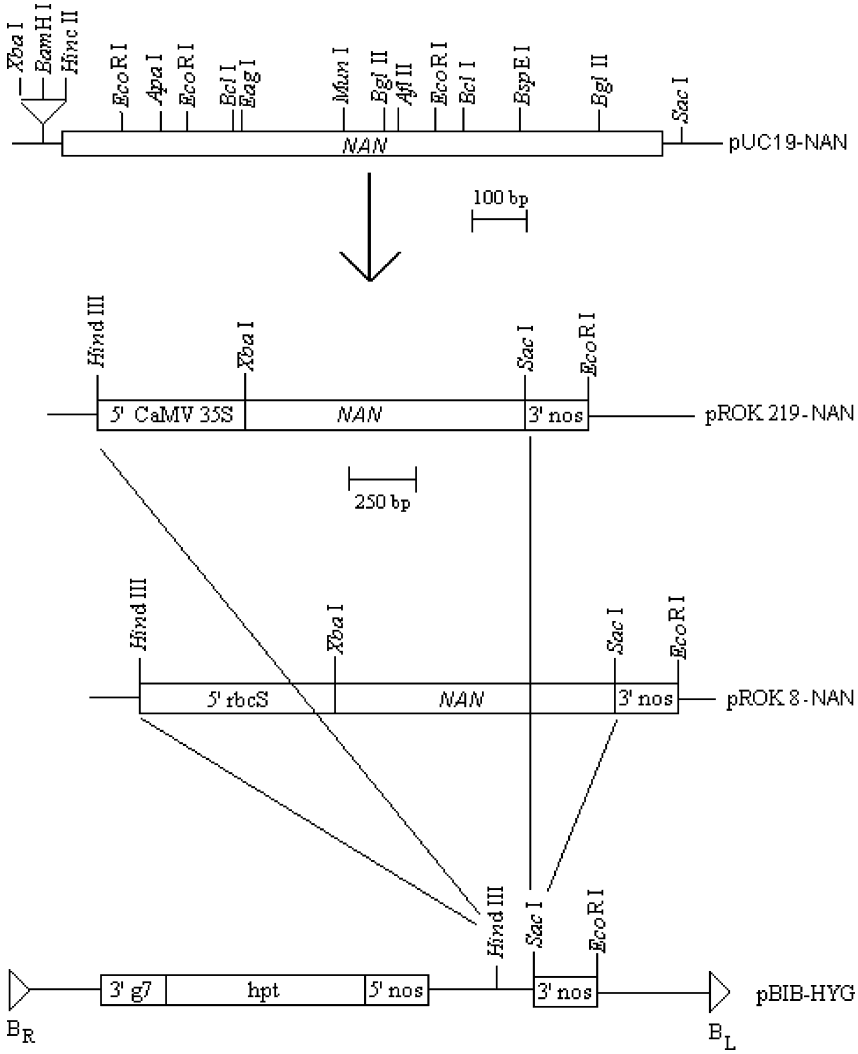

Figure 3. Construction and structure of *NAN* expression vectors.
The *NAN* gene was excised from pUC19-NAN using *Xba*I and *Sac*I, and cloned into expression cassettes under the transcriptional control of the CaMV35S and *rbcS* promoters in pROK219 and pROK8, respectively. The promoter-*NAN* fragments were then excised using *Hind*III and *Sac*I, and recloned into the binary vector pBIB-HYG, which contains a hygromycin resistance gene (*hpt*) for selection of transgenic shoots (Becker, 1990). nos, Polyadenylation signal derived from the nopaline synthase gene; B_R and B_L, T-DNA borders.

line in which reporter gene activity was confined almost entirely to root tissue, and an rbcS-NAN line which showed high levels of NAN expression in green tissues is shown in Figure 4(b,e), respectively. NAN activity was also localized in individual cells (Figure 4g,h). Cleavage of X-NeuNAc by NAN yields the same indigo blue pigment produced when X-Gluc is cleaved by GUS. Consequently, although X-NeuNAc and X-Gluc can be used individually to visualize the activity of one or other reporter gene in a given tissue sample, they cannot be combined for simultaneous detection of NAN and GUS. Fortunately, alternative commercially available substrates have been developed for the histochemical detection of GUS, for example, the indole derivative 5-bromo-6-chloro-3-indolyl β-D-glucuronide (X-GlucM), which produces a magenta chromophore; and 2-(2,4-hydroxy-3-methoxyphenyl) vinyl-1-methylquinolinium-β-D-glucuronide (VQM-β-D-glucuronide), which produces an intense red chromophore (Imlau *et al.*, 1999). We used X-GlucM to localize GUS activity in transgenic plants containing a transcriptional fusion between GUS and the promoter of a tobacco chlorophyll *a*/*b*-binding protein gene (Cab-GUS plants; Kavanagh *et al.*, 1988) or the promoter of an *Arabidopsis* β-amylase gene (Bmy-GUS plants; Lao *et al.*, 1999). GUS activity was detected in all tissues of Bmy-GUS seedlings (Figure 4d), but was mostly confined to cotyledons in Cab-GUS seedlings (Figure 4a). X-GlucM was found to be as sensitive a histochemical substrate as X-Gluc for visualizing GUS activity in plant tissues.

In order to investigate whether X-GlucM could be used in combination with X-NeuNAc for simultaneous detection of GUS and NAN activity, F_1 hybrid plants containing both reporter genes were produced by crossing the CaMV-NANr line with a Cab-GUS line (yielding CaMV-NAN × Cab-GUS plants) and by crossing an rbcS-NAN line with a Bmy-GUS line (yielding rbcS-NAN × Bmy-GUS plants). Seeds were recovered from both the selfed parental lines and the F_1 crosses, germinated on 1% agar and stained with X-GlucM and/or X-NeuNAc as appropriate. The reporter gene

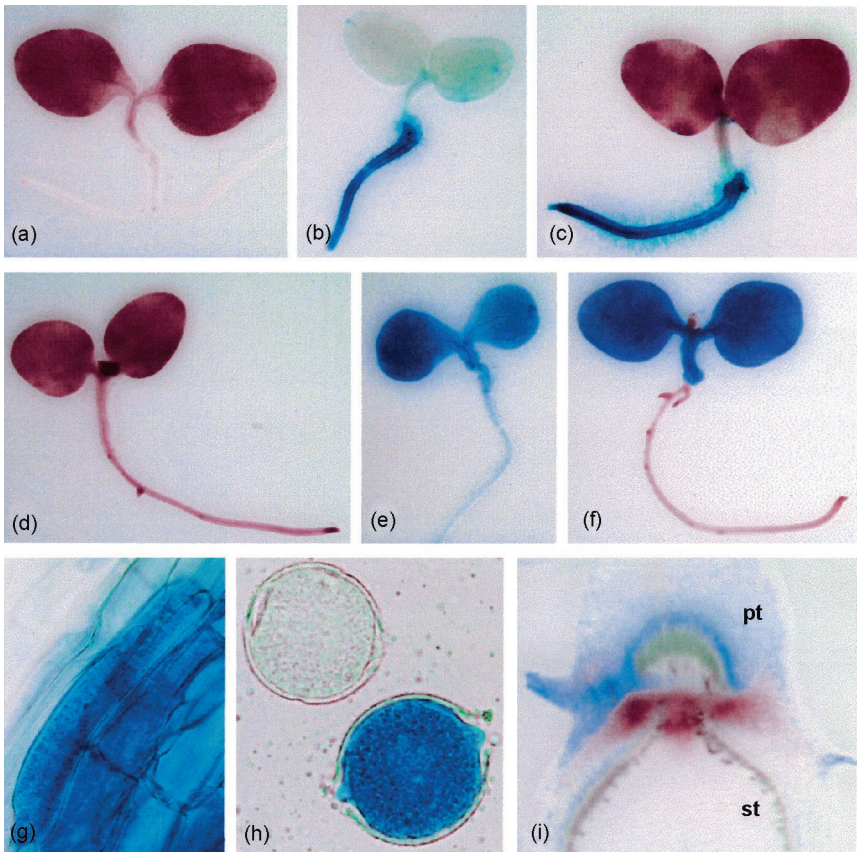

Figure 4. Histochemical detection of NAN and GUS in stably transformed plants.
NAN activity (blue stain) was localized using X-NeuNAc; GUS activity (magenta stain) was localized using X-GlucM.
(a) Cab-GUS seedling; (b) CaMV-NANr seedling; (c) simultaneous localization of NAN and GUS activity in a CaMV-NANr × Cab-GUS seedling; (d) Bmy-GUS seedling; (e) rbcS-NAN seedling; (f) simultaneous localization of NAN and GUS activity in an rbcS-NAN × Bmy-GUS seedling; (g) NAN activity in individual root cells of the CaMV-NANr line; (h) a stained and unstained pollen grain from an rbcS-NAN plant; (i) NAN and GUS activity in a transverse section through the stem (st) and attached petiole (pt) of a mature rbcS-NAN × Bmy-GUS plant. Magnifications: (a–f) ×5; (g) ×100; (h) ×400; (i) ×20.

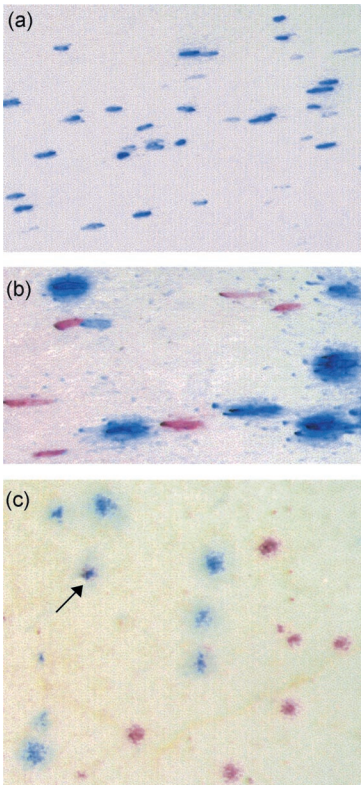

Figure 5. Histochemical detection of NAN and GUS following transient expression.
Plasmid DNAs containing a CaMV-NAN (pROK219-NAN) or a CaMV-GUS (pROK219-GUS-hpt) expression cassette were precipitated onto separate batches of 1.1 μm tungsten particles.
(a) Onion epidermal cells were bombarded with particles containing pROK219-NAN.
(b,c) Batches of tungsten particles containing pROK219-NAN or pROK219-GUS-hpt were mixed prior to delivery into onion epidermis (b) or tobacco leaf tissue (c).
NAN and GUS activity was visualized using X-NeuNAc and X-GlucM, respectively. Cells expressing NAN stain blue, those expressing GUS stain magenta. The arrow highlights a group of cells expressing both enzyme activities, which stained purple due to mixing of blue and magenta pigments. Magnifications: (a) ×50; (b) ×200; (c) ×400.

expression patterns observed in hybrid seedlings following incubation with both substrates (Figure 4c,f) corresponded precisely to a combination of the expression patterns observed in the original parental lines incubated with the relevant single substrates (Figure 4a,b,d,e). NAN and GUS activity was detected as blue and magenta staining, respectively, while co-expression resulted in a deep purple colour (e.g. in the cotyledons of an rbcS-NAN × Bmy-GUS seedling; Figure 4f). Both reporter activities were also visualized simultaneously in tissue sections (Figure 4i).

Histochemical localization of NAN and GUS activity in transiently transformed cells

The pUC19-based vectors pROK219-NAN (Figure 3) and pROK219-GUS-hpt (T.A.K., unpublished results), which contain a CaMV-NAN and a CaMV-GUS expression cassette, respectively, were used for transient expression studies in tobacco leaf and onion bulb epidermal cell layers. Each vector DNA was precipitated onto separate batches of tungsten microparticles that were either used alone or mixed together just prior to bombardment of the tissues. Following bombardment with pROK219-NAN-coated particles, NAN activity was visualized using X-NeuNAc (Figure 5a). Tissue samples bombarded with both plasmids were stained with X-NeuNAc and X-GlucM. Figure 5(b,c) shows discrete groups of onion epidermal and tobacco leaf cells, respectively, expressing NAN (blue-stained cells) or GUS activity (magenta-stained cells). Occasionally, cells expressing both enzyme activities could be identified. Such cells stained purple due to the mixing of the blue and magenta pigments (Figure 5c). We found that NAN, but not GUS, was detectable using 0.1 mM substrate, while both activities were clearly visible when 1 mM substrate was used. This suggests that GUS has a higher K_m for X-GlucM than NAN has for X-NeuNAc. In practice, the detection of GUS and NAN following transient expression in tobacco and onion cells is conveniently achieved using the same staining conditions.

Simultaneous detection of NAN and GUS activity in polyacrylamide gels

NAN (43 kDa) and GUS (68 kDa) were readily separated by denaturing or non-denaturing PAGE due to the magnitude of their molecular mass difference (25 kDa). Moreover, both enzymatic activities were detected *in situ* in polyacrylamide gels either following SDS–PAGE and a subsequent renaturation step (Schwarz *et al.*, 1987), or alternatively by non-denaturing PAGE without further treatment, by incubating the gel in GUS buffer containing both MUN and MUG (Berg *et al.*, 1985; Kavanagh *et al.*, 1988). Cleavage of either substrate produced an intensely fluorescent MU band under long-wave UV illumination corresponding to the location of the respective enzyme activity (Figure 6). NAN and GUS activities were also localized in polyacrylamide gels using X-NeuNAc and X-GlucM (data not shown).

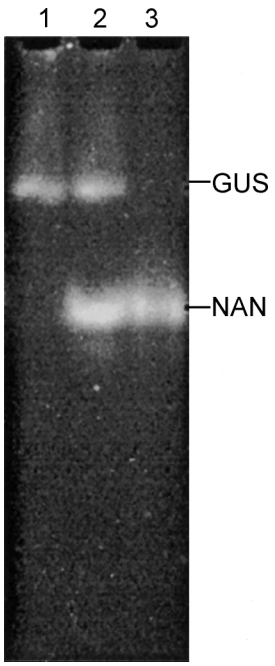

Figure 6. Detection of NAN and GUS in polyacrylamide gels.
Total protein extracts of a CaMV-NAN and a CaMV-GUS plant were electrophoresed in a non-denaturing polyacrylamide gel. Reporter enzyme activities were detected by incubating the gel in GUS buffer containing 0.05 mM MUN and 0.1 mM MUG. Lane 1, protein extract containing GUS activity; lane 3, protein extract containing NAN activity; lane 2, a 50 : 50 mixture of the GUS- and NAN-containing extracts used in lanes 1 and 2.

Simultaneous assay of NAN and GUS activities in cell-free extracts

Since NAN and GUS do not require different extraction or assay buffers, it is convenient to prepare cell-free extracts in GUS buffer and assay each activity in parallel using MUN and MUG, respectively. The possibility of detecting both enzyme activities in a single reaction was also investigated using MUN as a NAN substrate and resorufin β-D-glucuronide (ReG) as a GUS substrate. As enzymatic cleavage of each of these substrates releases a different fluorescent product, it should in theory be possible to quantify both enzyme activities in the same reaction. The K_m for GUS activity using ReG as the substrate was determined to be 0.15 mM, and this concentration was used in subsequent reactions. A protein extract containing both enzyme activities was first assayed in GUS buffer containing a single substrate, MUN or ReG. The NAN and GUS activity data obtained in these single-substrate assays was compared with that obtained when the assay contained both substrates (dual substrate assay). GUS activities were

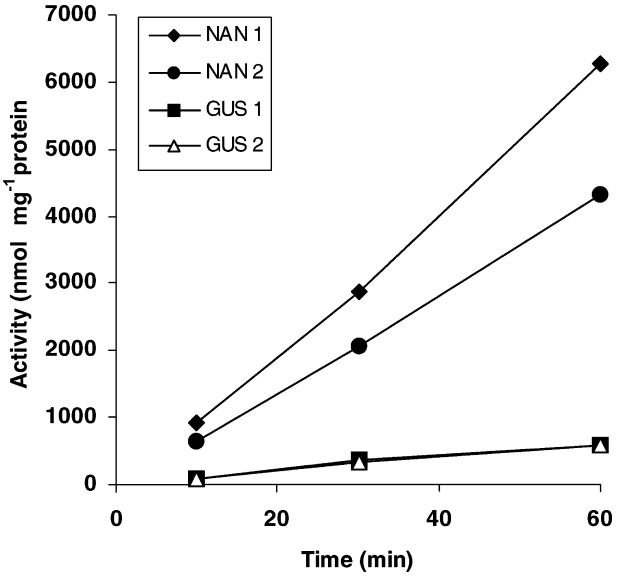

Figure 7. Simultaneous versus independent assay of NAN and GUS activity. NAN and GUS activities were determined using the substrates MUN and ReG, respectively. Enzyme activities were measured either independently in single substrate assays (NAN1, GUS1); or simultaneously in the presence of both substrates (NAN2, GUS2).

equivalent whether measured in the single or dual substrate assay, but NAN activity was apparently 30% lower when measured in the dual substrate assay (Figure 7). This discrepancy was shown to be due to absorption of MU fluorescence by the uncleaved ReG substrate (ReG-mediated quenching of MU fluoresence), and might be resolved by the use of a correction factor. An alternative strategy for simultaneous detection of NAN and GUS activities in the same sample might be to use substrates whose absorption and emission characteristics (and those of their cleavage products) do not overlap.

Experimental procedures

Construction of a synthetic NAN *gene*

The nucleotide sequence of the synthetic gene *NAN* was based on the amino acid sequence of the 'small' cytoplasmic (non-secreted) sialidase encoded by the *nan*H gene of *C. perfringens* A99. The gene was synthesized in three sections, the first of which, from *Bam*HI to *Apa*I, was constructed from six 5′-phosphorylated PAGE-purified 80-mer oligonucleotides (Genemed Biotechnologies, San Francisco, CA, USA). These were annealed in complementary pairs (75 pmol each oligonucleotide) in a solution containing 50 mM Tris–Cl (pH 7.9), 100 mM NaCl and 10 mM $MgCl_2$, by heating to 90°C and allowed to cool slowly to room temperature. The resulting double-stranded products possessed eight base 5′ overhangs and were ligated together using T4 DNA ligase. The corresponding full-length DNA fragment was then amplified from the ligation mixture by PCR using flanking primers, digested with *Bam*HI and *Apa*I, and cloned into pSL1190 (Pharmacia, Uppsala, Sweden). The second section, from *Apa*I to *Afl*II, was constructed in a similar fashion using eight 110-mer oligonucleotides that formed 20 base single-stranded overhangs when annealed in pairs. The third section, from *Afl*II to *Sac*I, was assembled by PCR extension of overlapping oligonucleotides as described by Stemmer *et al.* (1995), using 28 standard quality 40-mers (MWG, Ebersberg, Germany). The full-length 590 bp product was recovered from a PCR reaction which contained all 28 oligonucleotides by a second round of PCR with flanking primers. This product was digested with *Afl*II and *Sac*I, ligated with the other *NAN* gene fragments and the full-length ORF cloned into pUC19.

Expression of the NAN *and GUS enzymes in* E. coli

The *NAN* and GUS reporter genes were cloned into the vectors pET3a and pET24d (Novagen, Madison, WI, USA), respectively, for high-level expression mediated by phage T7 RNA polymerase. *NAN* was modified by PCR to place an *Nde*I site at the translation start site and a *Bam*HI site immediately after the stop codon, and cloned into pET3a. GUS was cloned directly from pRAJ275 into the *Nco*I and *Eco*RI sites of pET24d. Each construct was transformed into *E. coli* BL21 (DE3). Enzyme expression was induced in overnight cultures containing 1 mM isopropyl-α-D-galactoside, and total soluble protein lysates were prepared from the recovered cell pellets. The concentration of NAN and GUS in the lysates was determined following SDS–PAGE by comparing their respective band intensities with equivalent-sized protein markers of known concentration in Coomassie blue-stained gels. Protein concentrations were determined as described by Bradford (1976). Approximately equal quantities of the NAN and GUS proteins were used for the determination of their respective K_m and V_{max} values.

Enzyme kinetic measurements

NAN assays were carried out in solution using MUN (Sigma, St. Louis, MO, USA) as the substrate. GUS activity was measured using MUG (Sigma) or ReG (Sigma). Triplicate reactions were typically carried out in a volume of 0.5 ml GUS buffer (Jefferson *et al.*, 1987) at 37°C for 60 min, and 50 μl aliquots were removed at 0, 30 and 60 min, terminated by the addition of 3 ml 0.33 M Na_2CO_3, and measured against MU or RE standards on a Perkin Elmer LS-50B Luminescence Spectrophotometer (excitation at 365 nm, emission at 445 nm for MU and 571/585 nm for RE). K_m and V_{max} values were determined according to the Michaelis–Menten equation by assay with substrate concentrations increasing from 0.1 to 1.0 mM, and a minimum substrate to enzyme molar ratio of 10^6.

Plant transformation

Derivatives of the binary vector pBIB-HYG (Becker, 1990) containing various *NAN* gene expression cassettes were introduced into competent *Agrobacterium tumefaciens* LBA4404 cells by heat shock as described (Höfgen and Willmitzer, 1988). The resulting *A. tumefaciens* clones were used to transform leaf explants of *N. tabacum* var. Samsun, as described by Horsch *et al.* (1984). Transgenic shoots were selected on shoot regeneration medium (Murashige and Skoog, 1962) agar containing 40 μg ml^{-1} hygromycin.

Transient expression of NAN *and GUS in plant cells*

Transient expression studies were performed in tobacco leaf and onion epidermal monolayers using the Bio-Rad PDS-100/He Particle Delivery System (Bio-Rad, Hercules, CA, USA). Plasmid DNAs were prepared using a Qiagen Plasmid Midi Kit (Qiagen, Crawley,

UK) and quantified by UV spectrophotometry. Tungsten particles (1.1 μm) were prepared and coated with plasmid DNA (3 mg particles : 5 μg DNA) according to the manufacturer's protocol. Tissue samples were placed on a 1% agar plate at a distance of 9 cm from the microcarrier launch assembly. Macrocarriers were loaded with the DNA-coated particles, set at a distance of 0.25 inches from the rupture disk (rupture pressure 1100 psi), and bombardments were carried out under a vacuum of 28 inches Hg. The Petri dish was then sealed and the tissue incubated in the dark at room temperature for 24–48 h before histochemical or microscopic analysis.

Histochemical localization of NAN and GUS activity

Histochemical staining reactions were carried out in GUS buffer (Jefferson *et al.*, 1987) containing 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, and 1 mM X-GlucM (Glycosynth, Cheshire, UK) and/or 0.5 mM X-NeuNAc (Sigma). Tissue samples were first washed briefly in GUS buffer, then the substrates were introduced by vacuum infiltration for 1 min. Staining reactions were carried out for various times from 20 min to overnight at 37°C. Chlorophyll was subsequently removed from tissue samples by soaking briefly in 50% ethanol and then overnight in 80% ethanol. Photographs were taken using a digital Olympus DP10 camera mounted on an Olympus SZX9 light microscope.

Detection of NAN and GUS activity in polyacrylamide gels

NAN and GUS activity was detected *in situ* in polyacrylamide gels following separation by non-denaturing PAGE (Berg *et al.*, 1985; Kavanagh *et al.*, 1988) or alternatively by denaturing SDS–PAGE (with sample denaturation at 60°C), followed by the renaturation treatments described by Schwarz *et al.* (1987). Non-denaturing mini-gels comprising 8% polyacrylamide, 0.375 M Tris–Cl (pH 7.5) were prepared without a stacking gel. Running buffer and samples were prepared as described by Sambrook *et al.* (1989), and gels were electrophoresed at 140 V for approximately 3 h. Gels were soaked in GUS buffer for 10 min at room temperature and then incubated at 37°C for approximately 30 min in GUS buffer containing 0.1 mM MUG and 0.05 mM MUN. Fluorescent MU bands were photographed on a UV transilluminator using Polaroid 665 film and a Wratten 2E filter.

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