The *Gibberella fujikuroi* *niaD* gene encoding nitrate reductase: isolation, sequence, homologous transformation and electrophoretic karyotype location

Bettina Tudzynski,¹ Katrin Mende,¹ Klaus-Michael Weltring,¹
James R. Kinghorn² and Shiela E. Unkles²

Author for correspondence: Bettina Tudzynski. Fax: +49 251 83 3823.

¹ Institut für Botanik und Botanischer Garten, Westfälische Wilhelms-Universität, D-48149 Münster, Germany

² Plant Science Laboratory, Sir Harold Mitchell Building, School of Biological and Medical Sciences, University of St Andrews, Fife KY16 9TH, UK

The *Gibberella fujikuroi* *niaD* gene, encoding nitrate reductase, has been isolated and used to develop an efficient homologous transformation system. A cosmid vector designated pGFniaD was generated based on *niaD* selection and shown to give comparable transformation efficiencies. Using pGFniaD, a genomic library was prepared and used for genetic transformations, giving frequencies of up to 200 transformants per µg DNA. Of 15 transformants analysed by Southern blots, six showed homologous integration whilst the remaining nine integrated at heterologous sites, indicating that the vector may be used reliably for both types of integration. The system therefore may be used both for self-cloning of gibberellin biosynthetic genes on the basis of complementation of defective mutants, and also for gene disruption experiments. Electrophoretic karyotype determination suggested at least 11 chromosomes ranging from 2 to 6 Mb, the total genome size being at least 37 Mb. The *niaD* gene was assigned to chromosome V by Southern blot analysis. The *niaD* gene is interrupted by one intron, and remarkably the promoter sequence, but not the 3' untranslated sequence, is highly homologous to that of the corresponding *Fusarium oxysporum* gene. This situation appears to be unique with respect to the promoter regions of corresponding genes in related species of filamentous fungi.

Keywords: *Gibberella fujikuroi*, *niaD* gene, homologous transformation, electrophoretic karyotype, gene structure

INTRODUCTION

The ascomycetous filamentous fungus *Gibberella fujikuroi* is used to produce gibberellins (Bu'Lock *et al.*, 1974), fungal hormones which are used commercially to elevate growth in higher plants, including agriculturally and horticulturally important species. In the fungus, gibberellins appear to promote elongation of hyphae and asexual spore germination. The gibberellins are complex structures: diterpenoids with a characteristic ring system. Gibberellins of commercial value, GA₃, GA₄ and GA₇, are used in combinations as it is uneconomic to separate these molecules on an industrial scale (Vass & Jeffries, 1979; Brückner & Blechschmidt, 1991a, b). Although the

fungal biosynthetic pathway for such molecules has been studied and reasonably well characterized biochemically (Pitel *et al.*, 1971a, b; Hedden *et al.*, 1978), there is less information regarding their genetics and molecular biology. The rather limited classical genetic research has yielded several gibberellin-non-producing mutants (Bearder, 1983; Avalos *et al.*, 1994; Candau *et al.*, 1991, 1992). Progress in the molecular genetics of *G. fujikuroi* has been made recently by the isolation of DNA clones encoding 3-hydroxy-3-methyl glutaryl-coenzyme A reductase, farnesyl pyrophosphate synthase and geranyl-geranyl pyrophosphate synthase (B. Tudzynski and co-workers, unpublished) using DNA probes from other species. Other potential gibberellin biosynthetic genes have been isolated by differential display (Appleyard *et al.*, 1995).

To further the study and understanding of the role played by these genes in gibberellin biosynthesis and to facilitate

Abbreviation: GA, gibberellic acid.

The EMBL accession number for the sequence reported in this paper is X90699.

the isolation of further gibberellin-specific genes by complementation of GA-defective mutants, an efficient transformation system is required. In this regard, several attempts to develop genetic transformation have been carried out. Such studies report heterologous systems which yield low transformation frequencies of around 1–10 transformants per μg DNA (Sanchez-Fernandez *et al.*, 1991; Brückner *et al.*, 1992).

The aim of the work described here was to establish a reliable, efficient transformation system for *G. fujikuroi* and to obtain basic biological information about this important industrial fungus. We report the first homologous transformation for *G. fujikuroi* based on *niaD*, encoding nitrate reductase, its electrophoretic karyotype location and basic information on gene organization in *G. fujikuroi*.

METHODS

Strains and media. *G. fujikuroi* wild-type strain m567 was originally isolated from rice as a plant pathogen; it was obtained from the fungal strain collection, Weimar, Germany. The first *G. fujikuroi* mutant strain used in this study for transformation experiments was *niaD57*, isolated following ultraviolet mutagenesis of wild-type cells on medium containing 60 g $\text{NaClO}_3 \text{ l}^{-1}$, with 10 mM glutamate as a sole nitrogen source (Cove, 1976; Klittich & Leslie, 1988). It was classified as a *niaD* mutant as judged by its growth responses on various nitrogen sources. A second *niaD* mutation (*niaD10*) was generated in strain B1-41a to give strain B1-41a/10. Both strains (B1-41a and B1-41a/10) are blocked in kaurene oxidase, the first step of gibberellin biosynthesis (Bearder, 1983).

Standard DNA manipulation procedures. Strains were grown on *Gibberella* complete medium as described by Brückner *et al.* (1992). Genomic DNA and DNA from lambda phage was isolated according to standard procedures (Sambrook *et al.*, 1989) and plasmid DNA following the Qiagen protocol.

Electrophoretic karyotype determination. The method used was basically that of Smith *et al.* (1987) and Brody & Carbon (1989). Aliquots containing 10^8 protoplasts were embedded in low-melting-point agarose and their chromosomes separated by PFGE using Biometra Rotaphor electrophoresis equipment. The following chromosomal separation conditions were employed: 2 h, 600 s constant switching interval 140–150° log, 48 V, speed 5; 200 h, 9000–500 s decreasing switch interval, 110–98° log, 48–42 V, 10 °C. DNA was blotted on to Hybond nylon membrane and Southern blot analysis was performed using the 8.8 kb *Hind*III DNA fragment (Fig. 1a) carrying the *niaD* gene of *G. fujikuroi*. Prehybridization (5 h), hybridization (14 h) and washing ($2 \times \text{SSC}$, 0.1% SSC) were performed at 60 °C in a shaking water bath. Filters were exposed using Kodak X-ray film at –70 °C for 7 d.

Transformation procedure. The procedure for generation of protoplasts and transformation was essentially that described by Brückner *et al.* (1992). Protoplasts incubated with the various

plasmids and cosmids were added to molten Czapek-Dox agar to which 0.8 M KCl had been added as an osmoticum (10 mM nitrate was the sole nitrogen source). Plates were incubated at 28 °C for 5–7 d. Nitrate-utilizing colonies were subcultured on standard (e.g. non-stabilized) Czapek-Dox medium. The recipient strain used for the vector pJN1 was *niaD57*, whilst the recipient strain for the cosmid bank, pGFniaD, was *niaD10*.

Construction of a *G. fujikuroi* cosmid vector and genomic cosmid library. A 0.4 kb *Pst*I fragment containing the lambda cohesive ends was cloned into the *Pst*I site in the polylinker of vector pJN1 and the construct designated pGFniaD. A library was constructed using DNA isolated from the wild-type (strain m567) inserted into pGFniaD. Complete digestion of the vector and partial digestion of genomic DNA was carried out using *Eco*RI, and the DNA was ligated and packaged using a standard kit (Amersham). The library was stored as 11185 individual *Escherichia coli* colonies.

RESULTS

Isolation and subcloning of the *niaD* gene

A library of a partial *Sau*3AI genomic DNA from strain m567 was generated in vector lambda EMBL3. This library, containing 25000 p.f.u., was screened by DNA:DNA hybridization with pSTA8 as a probe carrying the *Aspergillus nidulans niaD* gene (Johnstone *et al.*, 1990). Twelve hybridizing lambda clones were revealed and their DNAs digested with a range of restriction enzymes. All twelve lambda clones were transformed into mutant strain *niaD57*. Lambda clone 11 gave the highest frequency of nitrate-utilizing transformants, yielding 12–15 transformants per μg DNA. After Southern blotting, the 8.8 kb *Hind*III fragment from lambda clone 11 which showed hybridization to the probe was subcloned into pUC19. This vector was designated pJN1 (Fig. 1a).

Gene and protein structure

The 8.8 kb *Hind*III fragment of pJN1 containing the *G. fujikuroi niaD* gene (Fig. 1a) was partially sequenced. The nucleotide sequence and inferred amino acid sequence of the region containing *niaD* are shown in Fig. 1(b). The protein sequence is highly homologous to many other nitrate reductases (reviewed by Campbell & Kinghorn, 1990), with haem-binding, flavin reductase and molybdenum cofactor domains. Overall, the *G. fujikuroi* deduced amino acid sequence shows the following homology with other fungal nitrate reductases: 94.5% to *Fusarium oxysporum* (Diolez *et al.*, 1993), 58.8% to *Leptosphaeria maculans* (Williams *et al.*, 1994), 58.7% to *Neurospora crassa* (Okamoto *et al.*, 1991), 54.4% to *Aspergillus nidulans* (Johnstone *et al.*, 1990) and 36.2% to *Ustilago maydis* (Banks *et al.*, 1993). The *G. fujikuroi* gene is interrupted by only one putative intron, interrupting the

Fig. 1. (a) Restriction endonuclease map of the 8.8 kb *Hind*III fragment of pJN1 containing the *G. fujikuroi niaD* gene (hatched) which was inserted into the *Hind*III site of pUC19 (not shown). The putative intron is indicated as a black box. (b) Nucleotide and inferred amino acid sequence of the *G. fujikuroi niaD* gene and its flanking regions. The putative intron is indicated in lower-case and by a break in the inferred amino acid sequence. 5' and 3' splicing sequences are underlined. The putative initiation codon is indicated in bold, as is the translational stop codon. Putative receptor motifs for the regulatory proteins NIT2/AREA (GATA) and the NIT4/NIRA (CTCCGNGG) in the promoter region of the *niaD* gene are underlined. TATA and CAAT motifs are overlined. A potential polyadenylation signal is also overlined. The chevrons above the sequence indicate a CT-rich motif typical of eukaryotic promoters.

Table 1. Transformation frequencies using the *G. fujikuroi niaD* gene

The recipient strain for vector pJN1 was *niaD57* (derived from wild-type strain m567). The recipient strain for pGFniaD and the pGFniaD cosmid library was *niaD10* (derived from kaurene oxidase mutant B1-41a).

Vector	No. of transformants per µg DNA*
pJN1	198, 89, 159, 174
pGFniaD	121, 136, 97
pGFniaD cosmid library	141, 182, 102, 126, 82

*Each number represents the mean of three to five parallel transformations from individual consecutive experiments.

codon for amino acid residue 606. An identical situation exists only in *N. crassa* (Okamoto *et al.*, 1991) and *F. oxysporum* (Diolez *et al.*, 1993) in terms of intron number and position. The promoter region also includes a TATA box at -124 bp (relative to the proposed ATG), and five potential CAAT boxes, at -67, -76, -85, -206 and -275 bp. The *niaD* gene is regulated by a wide-domain regulatory protein encoded by the *nit-2* gene in *N. crassa* (Fu & Marzluf, 1990) or *areA* in *A. nidulans* (Kudla *et al.*, 1990) as well as the pathway-specific inducible protein encoded by *nit-4* in *N. crassa* (Fu *et al.*, 1989) and *nirA* in *A. nidulans* (Burger *et al.*, 1991). The receptor binding sites for a NIT2/AREA binding protein (GATA) are present at -8, -133, -331 and -587 bp. A receptor binding site for a NIT4/NIRA transcriptional factor (CTCCGNGG) is found in the *G. fujikuroi niaD* promoter at -311 bp. In the 3' non-translated sequence, a possible polyadenylation site located 295 bp from the proposed translational stop codon is observed.

Nucleotide sequence analysis of the upstream region shows remarkable homology (91.2%) with that of *F. oxysporum* (Diolez *et al.*, 1993) for over 780 bases upstream from the translational start. Further upstream, there is no significant homology and similarly, in the 3' non-coding region, significant homology does not exist.

Homologous transformation

Vector pJN1 containing the 8.8 kb *Hind*III insert harbouring the *G. fujikuroi niaD* gene was introduced into the *niaD57* mutant strain, derived from wild-type strain m567, and transformants selected by growth on medium with nitrate as a nitrogen source. The frequency of nitrate-utilizing colonies is given in Table 1. Frequencies as high as 200 transformants per µg DNA were observed and such frequencies were considered to be sufficient for self-cloning experiments. Consequently a cosmid vector was generated and its ability to transform the *niaD10* strain (strain B1-41a/10 derived from a gibberellin-non-producing mutant) was confirmed (Table 1). Finally a genomic library was generated in vector pGFniaD. The

transformation frequencies using the cosmid library in pGFniaD in the *niaD10* mutant background were similar and again sufficient for self-cloning. Transformation levels for both *niaD* strains were between 80 and 200 transformants per µg DNA. As the reversion frequency of both *niaD57* and *niaD10* mutants was low, less than 1 in 1000 nitrate-utilizing colonies, it is therefore likely that nitrate-utilizing colonies obtained in transformation experiments are indeed *bona fide* transformants.

Putative transformants were analysed at the molecular level. Initially ten nitrate-utilizing transformants of *niaD10* produced using pGFniaD were examined by Southern blot hybridizations (data not shown). Of these, four showed disruption of the resident *niaD* band, indicative of homologous integration; one appeared unchanged compared with the recipient, as would be expected for gene conversion; while in the remaining transformants complex band patterns were obtained, suggesting multiple heterologous integrations. Following transformation of the *niaD10* strain with the cosmid library, 15 colonies capable of growth on nitrate as sole nitrogen source were chosen at random for analysis of integration events by Southern blotting and hybridization (Fig. 2). The genomic DNA was digested with *Eco*RI and blots probed with the 2.6 kb *Pst*I fragment of pJN1, containing most of the *niaD* gene, or *Pst*I-digested pUC19. Using the *niaD* probe, the resident 7.0 kb *Eco*RI band was visible in the recipient strain. Only two transformants (T2 and T13) showed a change in mobility of this band, giving in its place two bands, one of which also hybridized to the pUC19 probe, indicating homologous integration at the *niaD* site. Transformants T5, T7, T8 and T9 contained the 7.0 kb *niaD*-hybridizing band, but there was no hybridization to the pUC19 probe. The reversion rate of the recipient strain is low, so it is likely that these strains represent double cross-over events, i.e. gene replacements, although this has not formally been proven. The remaining transformants, T1, T3, T4, T6, T10, T11, T12, T14 and T15, had the resident *niaD* band and in addition a band of 12.6 kb (this band is faint in Fig. 2 for T14 and T15), the size of the pGFniaD cosmid vector. As expected, this band also hybridized to pUC19. Since the cosmid inserts are *Eco*RI fragments, this pattern suggests integration at sites other than the *niaD* gene via the *Eco*RI library insert. Of the 15 transformants, therefore, six show homologous integration of cosmid DNA at the *niaD* site, while the remaining nine transformants have cosmid DNA integrated at ectopic (i.e. heterologous) sites.

Electrophoretic karyotype determination

To resolve and determine the number of *G. fujikuroi* chromosomes we used PFGE conditions which were suitable for the separation of the three *Schizosaccharomyces pombe* chromosomes (Smith *et al.*, 1987). The *G. fujikuroi* chromosomes were resolved into eight bands (Fig. 3). These were designated chromosomes I–VIII, ranging in size from 2 to 6 Mb. Chromosome I was around 6 Mb, chromosome II 5.7 Mb, chromosome III 5 Mb, chromosome IV 4.2 Mb. Chromosomes V, VI, VII and VIII

Fig. 2. Southern blot and DNA hybridization analysis of *niaD*⁺ transformants. Genomic DNA (5 µg) from representative transformants obtained using the pGFniaD cosmid library was cleaved with *Eco*RI, which cuts the vector pGFniaD once. Following Southern blotting, filters were probed with the 2.6 kb *Pst*I *G. fujikuroi niaD* fragment (a) and linearized pUC19 (b). Lanes T1–T15 are representative transformants; m567 and B1-41a (left-hand lanes) are the recipient strains.

Fig. 3. Separation of intact *G. fujikuroi* chromosomal DNA. Lanes 2, 3 and 4 have different concentrations of *G. fujikuroi* DNA. Lane 1 is commercially prepared chromosomal DNA from *Schizosaccharomyces pombe*. The sizes of the three *S. pombe* chromosomes are indicated on the left. Separated *G. fujikuroi* bands are tentatively given chromosome numbers I–VIII in descending order according to size as discussed in the text. Since the total *G. fujikuroi* genome size is not known, for example as determined by DNA:DNA reassociation analysis, the band values given in the text are only approximate. After electrophoresis the DNA was transferred to a filter and probed with the *G. fujikuroi niaD* gene (lane 5).

were less than 3.5 Mb in size. Their approximate sizes were: chromosome V, 3.2 Mb; chromosome VI, 3.0 Mb; and chromosomes VII and VIII, around 2.0 Mb. Chromosome V and chromosome VI showed higher relative intensity of ultraviolet fluorescence after ethidium bromide staining, suggesting that these bands represented more than one co-migrating chromosome, probably three in the case of chromosome V and two for chromosome VI. This would indicate that the chromosome number in *G. fujikuroi* strain m567 is at least 11. Unfortunately there is no classical genetic or cytological determination avail-

able to relate to this molecular karyotype. Given that *G. fujikuroi* has this (minimum) estimate of 11 chromosomes, the genome size would be at least 37 Mb, which is in line with genome sizes found in other ascomycetous filamentous fungi such as *A. nidulans* (31 Mb; Brody & Carbon, 1989). The 8.8 kb *Hind*III fragment of pJN1 containing the *G. fujikuroi niaD* gene was radiolabelled to probe filters of resolved *G. fujikuroi* chromosomes. The *niaD* probe hybridized to chromosome V. Recent work reported by Xu *et al.* (1995) on a number of strains of *G. fujikuroi*, but not including that used in this work (i.e. m567), indicates a haploid number of 12 chromosomes.

DISCUSSION

The results presented here report a significant increase in transformation frequency of *G. fujikuroi* using the homologous nitrate reductase (*niaD*) system rather than the heterologous systems reported previously (Sanchez-Fernandez *et al.*, 1991; Brückner *et al.*, 1992). Such frequencies will enable the self-cloning of gibberellin biosynthetic genes by complementation of gibberellin-defective mutants, including the kaurene oxidase defective strain used here. This notion is supported by the fact that transformation frequencies are similar (120–200 transformants per µg DNA) irrespective of whether the *niaD* vector alone or a cosmid genomic library is used, and independent of the genetic background of the recipient.

The fact that the *niaD* gene constructs have been shown to integrate at a relatively high frequency at homologous sites indicates that the system may be useful for gene disruption experiments. Several potential gibberellin genes have now been isolated (Appleyard *et al.*, 1995; B. Tudzynski and co-workers, unpublished) and it should now be possible to test their involvement or role in gibberellin biosynthesis.

The *niaD* gene itself shows similarity to previously described fungal nitrate reductase genes. As expected, it has potential receptor nucleotide motifs for the global nitrogen regulator, NIT-2/AREA (e.g. GATA), as well

as for the nitrate pathway-specific inducer, NIT-4/NIRA (i.e. CTCCGNGG). There is no indication, however, from hybridization or sequence analysis that the nitrite reductase gene *nirA* is tightly linked to *nirD* (B. Tudzynski, unpublished), as has been found for a number of fungi.

Surprisingly, we observed high homology in the promoter regions of the *G. fujikuroi* and *F. oxysporum* *nirD* genes. *G. fujikuroi* is the perfect stage of the anamorph *Fusarium* section *Liseola*, and while it may be expected that members of the same genus would have high similarity in the coding regions of genes, it has not previously been reported that such high homology extends to the upstream regions. For example, other than the putative recognition motifs for regulatory proteins, there are no extensive stretches of nucleotide homology in the upstream sequences of the *Aspergillus nidulans*. *A. niger* (Unkles *et al.*, 1992) or *A. oryzae* *nirD* genes (S. E. Unkles, unpublished). Further analysis of gene structure in these organisms is required in order to establish the significance of this apparently unique arrangement.

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