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Isolation, characterization and disruption of the *areA* nitrogen regulatory gene of *Gibberella fujikuroi*

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Abstract The gene *areA-GF*, a homologue of the major nitrogen regulatory genes *nit-2*, *areA*, *nre* and *NUT1* of *Neurospora crassa*, *Aspergillus nidulans*, *Penicillium chrysogenum* and *Magnaporthe grisea*, respectively, was cloned from the gibberellin (GA)-producing rice pathogen *Gibberella fujikuroi*. *areA-GF* encodes a protein of 972 amino acid residues which contains a single putative zinc finger DNA-binding domain that is at least 98% identical to the zinc finger domains of the homologous fungal proteins. The *areA-GF* gene has been shown to be functional in *N. crassa* by heterologous complementation of a RIP induced *nit-2* mutant. The transformation rate was nearly as high as in a homologous complementation control. Transformants were able to utilize nitrate and expressed a normally regulated nitrate reductase activity. To generate *areA-GF*$^-$ mutants, gene replacement experiments were performed using a linearized replacement vector carrying the hygromycin B phosphotransferase (*hph*) gene. The replacement of the zinc finger by the hygromycin cassette resulted in transformants which were unable to utilize nitrogen sources other than ammonium and glutamine, and gave significantly reduced gibberellin production yields. Complementation of such a mutant with the wild-type gene led to the full recovery of gibberellin production.

Key words Nitrogen regulation · *Gibberella fujikuroi* · *areA* gene · Gibberellin biosynthesis

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Introduction

The rice pathogen *Gibberella fujikuroi* (imperfect stage: *Fusarium moniliforme*) produces large amounts of gibberellins and causes the superelongation or "bakanae" disease (foolish seedlings) of its host plant. The gibberellins, which are natural plant hormones, regulate various stages of plant development, such as stem elongation and induction of flowering. They have found many applications in agriculture, horticulture and the brewing industry (Brückner et al. 1989).

The biosynthetic pathway for the gibberellins has been deduced by the determination of chemical structures, the use of defective mutants and labeled precursors, and the analysis of biotransformations (Bearder 1983). However, very little is known about the molecular genetics of this pathway. Cloning of the first structural genes for gibberellin biosynthesis (Tudzynski and Hölter 1998; Tudzynski et al. 1998) has opened new horizons for analysis of their regulation and will provide important tools for the improvement of biotechnological production of these plant growth stimulators.

The negative control of gibberellin biosynthesis by rapidly utilized carbon sources and by high levels of ammonium has been known for a considerable time, and was first reported by Borrow et al. (1961, 1964) and Bu'Lock et al. (1974). In some wild-type strains, gibberellin biosynthesis begins upon depletion of the nitrogen source about 3 days after the onset of mycelial growth (Candau et al. 1992). In the high-producing wild-type strain *G. fujikuroi* m567, the synthesis of gibberellins starts before depletion of ammonium. The yield is reduced to 20–25% in a medium containing a high concentration of ammonium, whereas growth is not affected (Brückner and Blechschmidt 1991a, 1991b). In the strain *G. fujikuroi* ATCC 12616, a low but significant level of *ent*-kaurene biosynthetic activity and of gibberellin production occurs during the exponential growth phase (Munoz and Agosin 1993). However, *ent*-kaurene and gibberellin biosynthesis reaches much higher levels

when exogenous ammonium or glutamine has been exhausted in the culture. When the conversion of ammonium into glutamine was inhibited by L-methionine-DL-sulfoximine, it was shown that glutamine alone had a repressive effect and therefore seems to be responsible for nitrogen repression of GA_3 synthesis (Munoz and Agosin 1993).

Nitrogen repression has been studied at the molecular level in *Aspergillus nidulans* (Caddick et al. 1994) and *Neurospora crassa* (Marzluf 1993, 1996); in both cases a positive-acting DNA binding protein (AREA, NIT2) effects the regulation of many structural genes involved in the utilization of secondary nitrogen sources. The corresponding nitrogen regulatory genes have also been isolated from *Penicillium chrysogenum* (*nre*; Haas et al. 1995) and *Magnaporthe grisea* (*NUT1*; Froeliger and Carpenter 1996). Very little is known, however, about nitrogen control of pathogenicity or virulence genes which are not directly related to nitrogen metabolism, or about genes involved in nitrogen-controlled secondary metabolite formation. It is well known that plant-pathogenic fungi have to respond to rapidly changing environmental conditions when they gain entry to plant tissue. The fungus has to adapt to nutrients available on or within the host. Recent results show that limitation of some nutrients may lead to the induction or derepression of specific pathogenicity genes. In the tomato pathogen *Cladosporium fulvum*, the avirulence gene *avr9*, coding for a peptide elicitor, is induced by nitrogen limitation (Van den Ackerveken et al. 1994). In the rice blast fungus *M. grisea*, the *MPG1* gene, encoding a hydrophobin which is important for appressorium formation, is also expressed under conditions of nitrogen or carbon limitation (Talbot et al. 1993). On the other hand, disruption of the *M. grisea NUT1* gene, a homolog of *areA* and *nit-2* had only little effect on infection efficiency (Froeliger and Carpenter 1996).

The genes involved in secondary metabolite production may also be under control of the positive-acting nitrogen regulator. The NIT2 protein of *N. crassa* and the NRE protein of *P. chrysogenum* bind strongly to the intergenic promoter region of the *pcbB* and *pcbC* genes at a single site that contains two closely spaced GATA sequences (Feng et al. 1995; Haas and Marzluf 1995).

Expression studies of genes of the general isoprenoid pathway in *G. fujikuroi*, recently isolated in our group (HMG-CoA reductase gene, Woitek et al. 1996; FPP synthase gene, Homann et al. 1996; GGPP synthase gene, Mende et al. 1997), showed that they are constitutively expressed. These results confirm previous studies by Nes et al. (1986), Avalos et al. (1988) and Rybakov and Bourd (1991). However, the high level of intracellular *ent*-kaurene biosynthetic activity under nitrogen-limiting conditions suggests that one of the targets of nitrogen regulation might be the *ent*-kaurene synthase gene, which codes for the first gibberellin-specific step in this pathway. Expression studies on the recently isolated *ent*-kaurene synthase gene and four other genes of the gibberellin pathway in *G. fujikuroi* clearly demonstrate that the transcript level drastically increases under conditions of ammonium limitation (Tudzynski and Hölter 1998). The increased expression of this gene is possibly mediated by a positive-acting nitrogen regulatory protein, homologous to the *Neurospora crassa* NIT2 protein, which induces the expression of many genes under conditions of nitrogen limitation (Fu and Marzluf 1990a, b).

In this study, we report the cloning of a nitrogen regulatory gene, *areA-GF*, from *G. fujikuroi*, which is homologous to *nit-2* and *areA*. The functional equivalence of this gene to *nit-2* was demonstrated by transformation of the *Gibberella areA-GF* gene into a *nit-2* mutant of *N. crassa* obtained by repeat-induced point (RIP) mutagenesis, and analysis of ability of transformants to utilize nitrate. Furthermore, we investigated the role of this gene in gibberellin biosynthesis in *G. fujikuroi* by gene replacement and complementation of $areA^-$ transformants with the wild-type *areA-GF* gene.

Materials and methods

Fungal strains and culture conditions

G. fujikuroi m567, a wild-type strain isolated from rice, was provided by the Fungal Culture Collection (Weimar, Germany). The wild-type strain *G. fujikuroi* IMI 58289 was kindly provided by E. Cerdá-Olmedo (Seville). This strain was used for transformation experiments because of the high transformation rates attainable.

The *N. crassa nit-2* RIP mutant (a complete loss of function mutant) was obtained by the RIP technique (Selker and Garrett 1988) and has a severely damaged *nit-2* gene.

For DNA isolation and protoplasting, *G. fujikuroi* m567 or IMI 58289 was incubated in 100 ml of complete medium (CM; Pontecorvo et al. 1953) at 28° C on a rotary shaker at 200 rpm for 3 days or 18 h, respectively. For analysis of gibberellin production, the fungus was grown in a liquid complete medium containing 60 g/l sunflower oil, 15 g/l corn-steep solids (Sigma), 0.5 g/l $((NH_4)_2SO_4$ and 0.1 g/l KH_2PO_4 for 7–10 days at 28° C on a rotary shaker (200 rpm).

Bacterial strains and plasmids

Escherichia coli strain DH5α was used for plasmid propagation. Vector pUC19 was used to clone DNA fragments carrying the *G. fujikuroi areA-GF* gene or parts of it. For gene replacement experiments, the 2.7-kb *Bam*HI-*Hin*dIII fragment carrying the hygromycin B resistance cassette was cut from the vector pGPC1 (Desjardins et al. 1992) and cloned into the *Bam*HI/*Hin*dIII sites of pareA-GF1.

DNA isolation

Lyophilized mycelium was ground into a fine powder with a mortar and pestle and dispersed (in the case of DNA for use in PCR) in extraction buffer as described by Cenis (1993). DNA for Southern hybridization experiments was prepared following the protocol of Doyle and Doyle (1990). Plasmid DNA was extracted using the Genomed plasmid extraction kit.

Screening of a genomic library

About 40,000 recombinant phages of the *G. fujikuroi* m567 genomic library (Tudzynski et al. 1996) were plated on *E. coli* strain LE 392 and screened by plaque hybridization as described

(Sambrook et al. 1989). Plaques were blotted onto Gene Screen nylon membranes (DuPont) according to the manufacturer's instructions. A [^{32}P]dCTP-labeled PCR fragment containing the zinc finger-encoding region of the *areA-GF* gene was used as the homologous probe. Hybridization and washing were done at 65° C. Putative positive phages were selected, plated and screened in a second round of hybridization. Phage DNA was isolated as described by Sambrook et al. (1989) and used for restriction analysis.

Southern analysis

For Southern analysis, genomic, plasmid or phage DNA was digested to completion with appropriate restriction enzymes, fractionated in 1.0% (w/v) agarose gels, and transferred to nylon membranes (N^+, Amersham) by vacuum blotting. DNA probes were random-primer labeled and hybridizations were carried out overnight at 65° C. The filters were washed at hybridization conditions (2 × SSC, 0.1% SDS, 65° C, followed by 0.1 × SSC, 0.1% SDS).

Northern analysis

Total *G. fujikuroi* RNA was isolated according to Chambers and Russo (1986). Northern analysis was carried out with 15 µg of total RNA per lane. The hybridization probe was a radioactively labeled 2.0-kb PCR fragment amplified using primers are1 and are3 (see below). An RNA ladder (Gibco-BRL) was used as a size marker.

PCR

Some 50 ng of *G. fujikuroi* m567 DNA was used as template for amplification of the zinc finger region of the *areA-GF* gene. The specific primers (40 ng), which were provided by H. Haas (Innsbruck), had the following sequences: are A, 5′-TGTACNAAYTGYTTYACNCA-3′ (corresponding to the amino acid sequence CTNCFTQ); are B: 5′-TTCTTPATNACPTCNGTYTT-3′ [(P = A OR G)] (corresponding to the peptide KKIVDTK). DNA amplification was performed in 50-µl mixtures using 2.5 units of Taq DNA polymerase (Super-Taq, P.H. Stehelin AG, Basel). The PCR reactions were carried out for 36 cycles of 1 min denaturation at 94° C, 0.5 min annealing at 50° C and 0.5 min synthesis at 72° C. The PCR product was precipitated with 0.3 M sodium acetate and 2 vols. of ethanol and cloned using a pGEM-T vector system (Promega).

In order to show that the *areA-GF* gene of *G. fujikuroi* is interrupted by introns, PCR was done with genomic and cDNA as templates using the following primers: are1, 5′-CGCGAGGACCAAGCACC-3′ and are2, 5′-GGTCATTCTGCGCCG 3′.

Fungal transformations

Preparation of protoplasts of *G. fujikuroi* was carried out as described (Tudzynski et al. 1996). Some 10^7 protoplasts of the fungus were transformed with 10 µg of the linearized replacement vector pareA-GFR. Transformed protoplasts were regenerated at 28° C in complete regeneration agar [(0.7 M sucrose, 0.05% yeast extract, 0.1% $(NH_4)_2SO_4$ containing 120 µg/ml hygromycin B (Calbiochem)] for 6–7 days. Single conidial cultures were established from hygromycin B-resistant transformants and used for DNA isolation and Southern analysis.

For complementation of *areA-GF* mutants with the intact *areA-GF* gene, the protoplasts of the mutant strains were transformed with 10 µg of the vector pareA-GF1. Transformed protoplasts were regenerated in a stabilized (0.8 M KCl) Czapek-Dox agar containing $NaNO_3$ as nitrogen source.

N. crassa transformation was done according to Cherniack et al. (1990). Briefly, 7-day-old conidia of the nit-2rip strain were germinated in Vogel's complete medium and subjected to Novozym 234 digestion to give spheroplasts. Transformants were selected on Vogel's-N medium supplemented with 20 mM sodium nitrate. Two µg of DNA and 100 µl of spheroplasts were used for each transformation, typically yielding 50–100 transformants.

Nitrate reductase assay

To assay nitrate reductase, mycelia from 50 ml cultures on Vogel's-N supplemented with 20 mM glutamine were transferred to three flasks, containing Vogel's-N plus 20 mM glutamine (nitrogen repression), 20 mM glutamine and 20 mM nitrate, or 20 mM nitrate. After 3 h of growth, mycelia were harvested and ground in liquid nitrogen to make whole cell extracts. Nitrate reductase was assayed as described (Garnett and Cove 1976).

Gibberellin analysis

For analysis of gibberellin formation, the wild-type strain and *areA-GF* disruptants were cultivated in test tubes containing 5 ml of Darken medium (Darken et al. 1959). The cultures were incubated for 7 days on a rotary shaker (200 rpm) at 28° C. GA_3 was analyzed by HPLC according to Barendse and van de Werken (1980) using a Merck HPLC system with a UV detector and a Lichrispher 100 RP18 column (5 µm, 250 × 4 mm; Merck). GA_3, GA_4, and GA_7 were also analysed by thin-layer chromatography using a mixture of ethylacetate: chloroform: acetic acid (60:40:5).

Results

Cloning of *areA-GF* a putative *G. fujikuroi* nitrogen regulatory gene

Two degenerate oligonucleotide primers, areA and areB, derived from the highly conserved zinc finger domain of the AREA protein of *A. nidulans* and the NIT2 protein of *N. crassa*, were used for PCR amplification. The resulting 150-bp PCR fragment (Fig. 1) was cloned into pGEM and sequenced. The nucleotide sequence showed 75% and the deduced amino acid sequence 98% identity to the zinc finger region of the corresponding *N. crassa* sequence. This fragment was subsequently used as a probe to screen an EMBL3 library of *G. fujikuroi*. Seven hybridizing plaques were detected and purified in a second round of screening. All clones showed a similar restriction pattern and yielded identical hybridizing bands with the 150-bp PCR fragment as probe. DNA from one of the seven selected phages, designated lambda are3, was used for subcloning of a 6.6-kb *Sph*I fragment and a 4.0-kb *Pst*I fragment into pUC19, resulting in the plasmids pareA-GF1 and pareA-GF2, respectively. Those plasmids were used to produce a physical map of the *areA-GF* gene and its 5′ and 3′ flanking regions (Fig. 1). Number and/or sizes of restriction fragments derived from this map correspond to the number and sizes of hybridizing bands seen in Southern blots of genomic DNA (data not shown), suggesting that the *areA-GF* gene is present in a single copy.

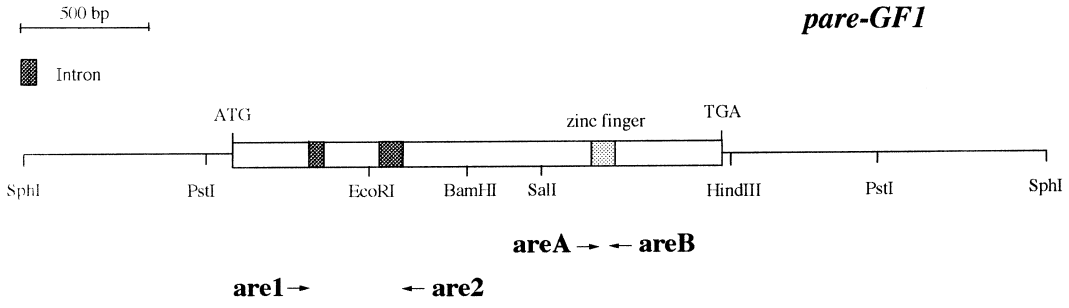

Fig. 1 Physical map of the plasmid pare-GF 1, carrying the *Gibberella fujikuroi areA* gene. The internal 4.0-kb *Pst*I fragment was used to construct plasmid pare-GF2. The *areA-GF* coding region is composed of three exons. The zinc finger is indicated by the *stippled box*. The Genbank accession for the *areA-GF* gene is Y11006. The positions of the PCR primers areA and areB (used for gene cloning) and the primers are1 and are2 (used for RT-PCR) are indicated

Sequence analysis

Sequence analysis of the coding and flanking regions of the *areA-GF* gene revealed three ORFs of 508 bp, 357 bp and 2050 bp. The deduced introns of 84 bp and 148 bp show 5′ and 3′ consensus splicing sites typical for filamentous fungi (Gurr et al. 1987). Both introns are located at the same positions as the two introns within the *N. crassa nit-2* gene. The existence of the proposed introns was proved by RT-PCR analysis with genomic and cDNA as templates, using the primers are1 and are2. The primers flank the introns and bind at the positions +353 and +1167 downstream of the translation start (Fig. 1). The expected fragments of 821 bp and 585 bp were amplified using genomic DNA and cDNA as templates, respectively (data not shown). Cloning of the cDNA fragment and sequence analysis confirmed the existence of the predicted introns.

The *areA-GF* gene encodes a protein of 972 amino acids with regions of considerable homology to NIT2, NUT1, NRE and AREA. An alignment of all known fungal nitrogen regulatory proteins revealed 71%, 66%, 61% and 59% identity between AREA-GF and NIT2, NUT1, NRE and AREA, respectively. A comparison of the relevant zinc finger regions between the AREA-GF, NIT2, NRE and AREA proteins is shown in Fig. 2A. In the zinc finger motif 49 out of 50 amino acids are identical in all five polypeptides. On the basis of the sequence alignment, a phylogenetic tree was constructed using the program TREE (HUSAR, Heidelberg). Figure 2B shows that the AREA-GF protein is most

A

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           681                                                                750
G.fujikuroi  AAGNGNDGNA PTTCTNCFTQ TTPLWRRNPE GQPLCNACGL FLKLHGVVRP LSLKTDVIKK RNRGSGTNVP
N.crassa     AAGNSTD..T PTTCTNCFTQ TTPLWRRNPD GQPLCNACGL FLKLHGVVRP LSLKTDVIKK RNRGSGASLP
M.grisea     Q.GNNQGGDA PTTCTNCATQ TTPLWRRNPE GQPLCNACGL FLKLHGVVRP LSLKTDVIKK RNRGSGSNVP
P.chrysog.   .......... PPACTNCFTQ TTPLWRRNPE GQPLCNACGL FLKLHGVVRP LSLKTDVIKK RNRSSANTLT
A.nidulans   .......... .TTCTNCFTQ TTPLWRRNPE GQPLCNACGL FLKLHGVVRP LSLKTDVIKK RNRNSANSLA

CONSENSUS                 **** ** *********  ********** ********** ********** *** *
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B

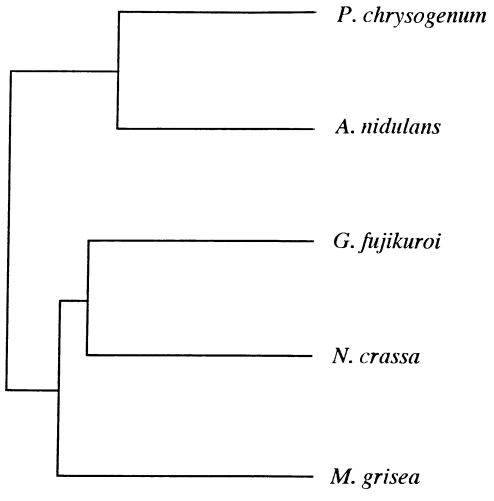

Fig. 2 A Partial sequence alignment of the zinc finger regions of the proteins AREA-GF (*G. fujikuroi*), NIT2 (*N. crassa*), NUT1 (*M. grisea*), AREA (*A. nidulans*), and NRE (*P. chrysogenum*). **B** Phylogenetic tree constructed on the basis of progressive alignment of the deduced sequences of AREA-GF (*G. fujikuroi*), NIT2 (*N. crassa*), NUT1 (*M. grisea*), AREA (*A. nidulans*) and NRE (*P. chrysogenum*)

closely related to NIT2 of *N. crassa* and NUT1 of *M. grisea*.

The 5′ non-coding region of the gene contains several pyrimidine-rich tracts. In addition, four potential AREA-binding GATA motifs were found (in both orientations) upstream of the *areA-GF* coding region. A typical eukaryotic polyadenylation site in the 3′ non-coding region was not found.

Regulation of the expression of *areA-GF* and *niaD-GF*

In other fungi, the expression of the *areA* gene is dependent on the nitrogen source. An excess of ammonium ion or glutamine, in contrast to nitrate and other nitrogen sources, leads to down-regulation of expression. In order to determine whether the *areA* gene of *G. fujikuroi* is regulated in a similar fashion, Northern experiments were done with RNA isolated from cells grown in the presence of glutamine for 2 days and then transferred to nitrate. After 2 days of further incubation, glutamine was added again to each culture. Figure 3 shows the level of *areA-GF* mRNA at different times in *G. fujikuroi* grown under these various conditions. Expression increased immediately after transferring the mycelium to a medium containing nitrate as the sole nitrogen source. The addition of glutamine rapidly repressed expression of the *areA-GF* gene: 5 min after glutamine was added there was a marked reduction in *areA-GF* mRNA levels, indicating repression of its synthesis and also suggesting that the pre-existing mRNA might be subject to rapid turnover. However, the *areA-GF* mRNA never entirely disappeared; this is typical for the effects of global regulators which interact with different pathway-specific factors, e.g. NIT2 with NIT4 in *N. crassa* (Marzluf 1996). The amount of *areA-GF* mRNA had already increased again after 60 min, probably due to the utilization of the added glutamine.

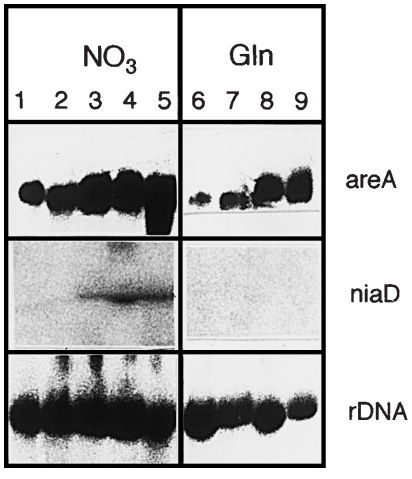

Fig. 3 Northern analysis of *G. fujikuroi* mRNA. Total RNA was extracted from mycelium grown on glutamine for 2 days. Mycelium was washed and then transferred to a medium containing 25 mM nitrate. Samples were taken after 0, 5, 15, and 60 min (lanes 2–5). After 2 days glutamine was added, to a final concentration of 25 mM, and samples were taken after 5, 15, 60, and 240 min (lanes 6–9). Lane 1 contains mRNA from mycelium grown on urea for 2 days. The RNA was probed with the *areA-GF* gene and the *G. fujikuroi niaD* gene. Hybridization to the small subunit of rDNA was used as a loading control

These data show that the level of *areA-GF* mRNA is significantly enhanced under nitrogen derepression conditions and is reduced under nitrogen repression conditions. Therefore, the expression of *areA* in *G. fujikuroi* is subject to nitrogen control, as has already been demonstrated for the corresponding *N. crassa nit-2* (Fu and Marzluf 1987), *A. nidulans areA* (Caddick 1992) and *P. chrysogenum nre* (Haas et al. 1995) genes.

Northern analysis of expression of the *G. fujikuroi niaD* gene, which encodes nitrogen reductase (Tudzynski et al. 1996), using the same growth conditions, showed a much more stringent control of expression: the *niaD* transcript level increased drastically in cells grown in a medium with nitrate as the only nitrogen source. Addition of glutamine led to the disappearance of *niaD* transcript within 5 min (Fig. 3).

Complementation of a *N. crassa nit-2* mutant with the *areA-GF* gene from *G. fujikuroi*

In order to demonstrate the function of the *areA-GF* gene directly, the plasmids pareA-GF1 (6.6-kb *Sph*I fragment) and pareA-GF2 (4.0-kb *Pst*I fragment), both carrying the complete coding region with different parts of the promoter (see Fig. 1), were used to complement a *N. crassa* RIP-induced mutant, carrying a loss of function mutation in the *nit-2* gene. As a positive control for transformation experiments, the wild-type *N. crassa nit-2* gene was transformed into the *nit-2* RIP mutant. Both plasmids, pareA-GF1 and pareA-GF2, transformed the *N. crassa* mutant with an efficiency equal to that obtained with the authentic *N. crassa nit-2*$^{+}$ gene (approximately 30 transformed colonies per μg DNA).

In order to show that the *G. fujikuroi* AREA-GF protein is able to turn on the expression of genes which are under control of NIT2, nitrate reductase activity was determined in several transformants. As shown in Table 1, the expression of nitrate reductase seems to be regulated as in the *N. crassa* wild-type strain: no activity or extremely low activity was present when transformants were grown in glutamine alone or in glutamine and nitrate, but high levels of enzyme activity were found in the presence of nitrate alone. Therefore, the *G. fujikuroi areA-GF* gene functions and controls expression of the NIT2-regulated nitrate reductase gene in *N. crassa* in a completely normal fashion. The results show clearly that the *G. fujikuroi areA-GF* and the *N. crassa nit-2* genes encode equivalent gene products.

Replacement of the *G. fujikuroi areA-GF* gene

In order to obtain *areA-GF* mutants via homologous integration of a mutated gene copy, the gene replace-

Table 1 Nitrate reductase activity in the *nit-2* RIP *N. crassa* host and transformants

Strain	Nitrate reductase activity in cells[a] cultured in the presence of		
	20 mM glutamine	20 mM glutamine +20 mM $NaNo_3$	20 mM $NaNo_3$
nit-2 RIP (host)	1	1	1
nit-2 RIP, transformed with pare-GF2	0	2	80
nit-2 RIP, transformed with pare-GF1	1	5	108
nit-2 RIP, transformed with the *N. crassa nit-2* gene	1	1	100

[a] The nitrate reductase activity was normalized with respect to protein concentration and is expressed in arbitrary units. See text for further details of culture conditions

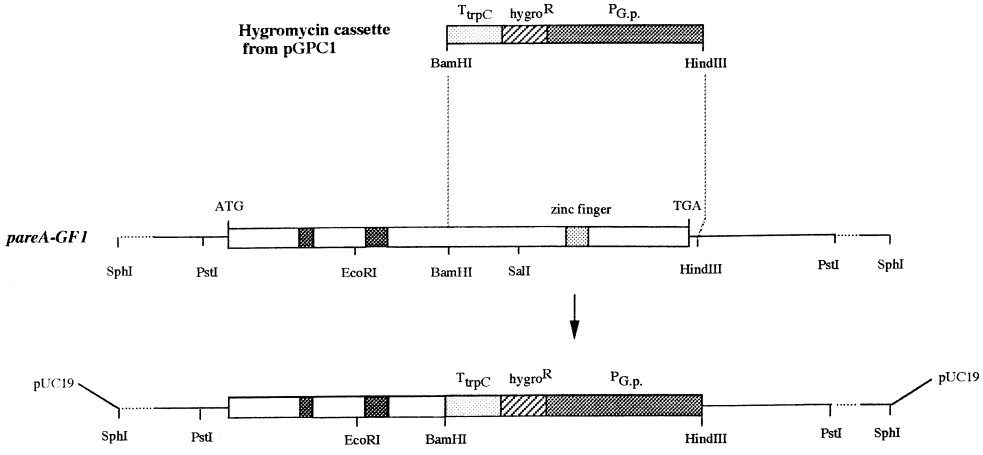

Fig. 4 Construction of the gene replacement vector pareA-GFR

ment vector pareA-GFR was constructed (Fig. 4). In *G. fujikuroi*, homologous integrations occur with a frequency of 20–30% (B. Tudzynski, unpublished results). The 1.5-kb *Bam*HI-*Hin*dIII fragment of the *areA-GF* gene carrying the zinc finger region was replaced by a hygromycin resistance cassette derived from the plasmid pGPC1 (Desjardins et al. 1992). Transformants were genetically purified by isolation of single spores. Among 52 transformants analyzed, three (T5, T6, and T19) had lost the ability to grow on nitrate and nitrite. Southern analysis of *Pst*I- and *Sph*I-digested DNAs from the transformants and the wild-type strain with the 5.4-kb *Sph*I-*Hin*dIII fragment of the plasmid pareA-GF1 (Fig. 1) showed that in the transformants the intact gene had been replaced by the linearized replacement vector lacking the zinc finger region (Fig. 5). Because the hygromycin cassette contains internal *Pst*I and *Sph*I sites the 4.0-kb wild-type band in the *Pst*I digest and the 6.6-kb wild-type band in the *Sph*I digest are replaced by smaller bands in the mutants.

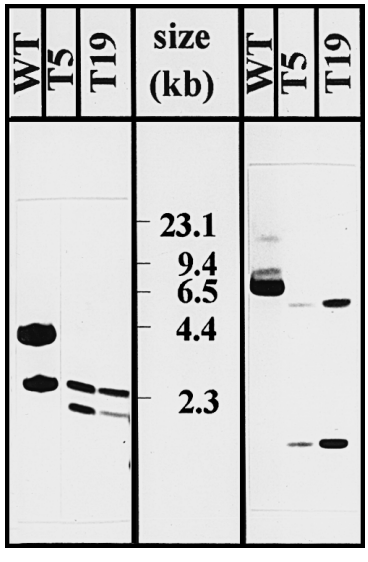

Fig. 5 Southern analysis of transformants obtained by transformation with the replacement vector pareA-GFR. Total genomic DNA from the wild-type strain and mutant transformants was digested with *Pst*I and *Sph*I and probed with the 5.4-kb *Sph*I-*Hin*dIII fragment of pareA-GF1. The sizes of the DNA marker bands are indicated

Gibberellin production by the *areA-GF*$^-$ mutants

Because gibberellin formation is dependent on the quality and quantity of the nitrogen source in *G. fuji-*

Table 2 Gibberellin production by the wild-type strain *G. fujikuroi* IMI 58289, *areA-GF*$^-$ mutants (T5, T6 and T19) and selected T19 transformants complemented with the *areA-GF* gene

Strain	GA_3[a] (mg/l)
IMI58289 (wild-type)	382, 420
T5	100, 90
T6	0, 0
T19	92, 85
T19/3	265, 310
T19/12	430, 440
T19/13	405, 469
T19/18	425, 462
T19/26	328, 395
T19/57	420, 372
T19/77	40, 65
T19/66	615, 625

[a] The gibberellin concentrations were analyzed by HPLC from two parallel shake flasks

kuroi, the possible influence of the *areA-GF* mutation on the yield of gibberellin was analyzed. The *areA-GF*$^-$ mutants T5, T6, T19, and the wild-type strain, were cultivated in a complete production medium. Gibberellin formation was analyzed by TLC and HPLC. Transformant T6 is not able to produce gibberellins at all, whereas strains T5 and T19 produced reduced amounts of GA_3 in comparison to the wild-type strain (Table 2). In an additional experiment, the wild-type strain and the mutants T5 and T19 were also cultivated in complete medium containing a tenfold higher amount of ammonium. In the wild-type strain the expected repressive influence of ammonium ion on gibberellin production could be demonstrated: only 15–20% of the GA_3 yield attained with the normal production medium, was found. In contrast, this ammonium-dependent difference in gibberellin yield could not be detected in the mutants T5 and T19, which produced approximately the same amount of gibberellins as the wild-type strain under conditions of ammonium repression, independently of the ammonium concentration in the medium.

In order to determine whether the loss of or reduction in the ability to produce gibberellins was due to the mutation in the *areA-GF* gene, the transformants T6 and T19 were complemented with the plasmid pareA-GF1 carrying the wild-type *areA-GF* gene. The ability to utilize nitrate as the only nitrogen source was used as a selection marker. Fifty complemented transformants of T6 and fifty of T19 were cultivated in the gibberellin production medium for gibberellin assays. None of the analyzed transformants of T6 was able to produce any gibberellins. Therefore, the total loss of gibberellin biosynthetic ability seems to be an additional effect of the replacement experiments in T6 and is not caused by the mutation in the *areA-GF* gene. On the other hand, most of the complemented transformants of T19 showed a restored ability to produce gibberellins in amounts comparable to those of the wild-type strain. The results of gibberellin analysis for some randomly selected transformants are shown in Table 2.

Discussion

The phenomenon of preferential utilization of favoured nitrogen sources by repression of enzymes and permeases involved in the utilization of nitrogen sources other than ammonium or L-glutamine is known as nitrogen metabolite repression (Wiame et al. 1985; Marzluf 1993). Recently it was shown that nitrogen metabolite repression controls not only genes encoding enzymes of primary metabolism, like *niaD* and *niiA*, but also regulates genes involved in fungus-plant interactions (Van den Ackerveken et al. 1994) and secondary metabolism (Feng et al. 1995; Haas and Marzluf 1995). Expression studies of a reporter gene encoding β-glucuronidase, under the control of the *P. chrysogenum acvA-pcbC* intergenic region, confirmed that indeed both genes are repressed by nitrogen (Feng et al. 1995). It was shown by mobility shift experiments that there is a strong interaction between the *P. chrysogenum* NRE-β-gal fusion protein and a site that contains two GATA motifs (Haas and Marzluf 1995). On the other hand, the *acvA-pcbC* intergenic region from *A. nidulans* – a species in which ACVS and PNS expression is not repressed by ammonium, contains only one single GATA motif (Peñalva et al. 1991).

Since gibberellin biosynthesis is also influenced by high concentrations of ammonium or glutamine, it is of general interest to learn more about the molecular mechanism of this nitrogen regulation. Therefore, we have cloned and characterized the major nitrogen regulatory gene *areA-GF* from *G. fujikuroi*, which obviously encodes a positive-acting regulatory protein homologous to the well known fungal proteins NIT2, AREA, NRE and NUTI (Fu and Marzluf 1990a, b; Kudla et al. 1990; Haas et al. 1995; Froeliger and Carpenter 1996). This confirms the report of Dickman and Leslie (1992), who showed that in *G. zeae*, another species of the genus *Fusarium*, a comparable gene must exist: they were able to complement the *G. zeae nnu* mutant with the *N. crassa nit-2* gene, restoring the ability to use several secondary nitrogen sources.

Northern analysis demonstrated that the expression of *areA-GF* is down-regulated by a high concentration of ammonium or glutamine. The fact that *areA-GF* expression could be detected even under nitrogen repression conditions leads to the conclusion that there might be another signal that mediates nitrogen repression in *Gibberella*. In *N. crassa*, another nitrogen metabolic regulator, the NMR protein, acts by binding to NIT2 and inhibits the latter's function, perhaps by preventing NIT2 DNA binding (Marzluf 1996). Thus, nitrogen repression in *G. fujikuroi* may also result from a combination of two effects, one of which is the transcriptional control of *areA-GF,* the other involving a specific protein-protein interaction. A gene with significant homology to the *N. crassa nmr* gene was recently isolated from *G. fujikuroi* (B. Tudzynski, unpublished results).

Interestingly, besides AREA homologs, fungi possess multiple GATA factors. *N. crassa* contains at least five distinct GATA factors: NIT2, which functions in nitrogen control; WC1 and WC2, which function in light perception (Ballario et al. 1996; Linden and Macino 1996); and two newly identified but clearly distinct GATA factors, one of which has two zinc fingers (B. Feng, L. Zhou, H. Haas, and G. A. Marzluf, unpublished results). Recently, a second GATA transcription factor (NREB) has been identified in *P. chrysogenum* (Haas et al. 1997). This protein shares several features with Dal 80p and Gzf3p, both negative acting GATA factors in nitrogen metabolism in *Saccharomyces cerevisiae* (Cooper 1996). In contrast to NRE and the other positive-acting GATA factors involved in nitrogen regulation in filamentous fungi, NREB appears to act as inhibitor of transcription. It is still not known how these multiple GATA factors, which share similar DNA binding specificities, regulate entirely different cellular functions in fungi.

Langdon et al. (1995) and Stankovich et al. (1993) have shown by N- and C-terminal deletions that a central region of the AREA protein of just 224 amino acid residues (amino acids 343-566) contains all elements essential for function, such as the DNA-binding domain, a signal for nuclear localization and an acidic activation domain. Therefore, gene replacement experiments with a vector lacking the zinc finger domain, were performed. Three out of 52 transformants were unable to utilize nitrate and nitrite and showed the hybridization patterns expected for the desired disruptants (Fig. 5). Two of them showed reduced gibberellin production, whereas one of the transformants (T6) lost its ability to produce gibberellins, altogether. This last phenotype does not seem to be due to the replacement of the *areA-GF* gene, since the other two mutants showed hormone formation (albeit very reduced); furthermore the ability to produce gibberellin could not be restored by complementation of T6 with pareA-GF.

Complementation of the transformant T19 led to the full recovery of wild-type gibberellin levels in the production medium, suggesting that gibberellin biosynthesis is subject to control by AREA-GF. Under conditions of ammonium repression (10 g/l $(NH_4)_2SO_4$), the wild-type strain produces reduced amounts of gibberellins (15–20% of the maximum yield in the production medium) only. Interestingly, the *areA-GF*$^-$ mutants also produced 15–20% of the maximum yield, irrespective of the ammonium concentration in the medium. Therefore, the physiological inhibition of the *areA-GF* gene by high ammonium concentrations affects the gibberellin yield in the same way as mutation of *areA-GF* by gene replacement.

Froeliger and Carpenter (1996) also isolated *M. grisea* NUT1 disruption tranformants which do not contain the zinc finger domain and are incapable of utilizing nitrate, but are still able to utilize some other nitrogen compounds, such as proline and alanine. The disruption mutant was still pathogenic on rice, although the lesions produced were somewhat reduced in size (Froeliger and Carpenter 1996). Lau and Hamer (1998) have isolated other mutants of *M. grisea* altered in nitrogen metabolism (via chlorate resistance) which show a loss of pathogenicity. Therefore, for *M. grisea* (Froeliger and Carpenter 1996), and possibly also for *G. fujikuroi*, additional nitrogen control systems may exist that function independently of NUT1 and AREA-GF. On the other hand, for *G. fujikuroi* one can assume that besides being subject to the global nitrogen regulation system gibberellin production is under the control of a positive-acting, pathway-specific regulator.

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