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Functional analysis of the *nifQdctA1*y4vGHIJ operon of *Sinorhizobium fredii* strain NGR234 using a transposon with a NifA-dependent read-out promoter

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Rhizobia are a disparate collection of soil bacteria capable of reducing atmospheric nitrogen in symbiosis with legumes (Fix phenotype). Synthesis of the nitrogenase and its accessory components is under the transcriptional control of the key regulator NifA and is generally restricted to the endosymbiotic forms of rhizobia known as bacteroids. Amongst studied rhizobia, *Sinorhizobium fredii* strain NGR234 has the remarkable ability to fix nitrogen in association with more than 130 species in 73 legume genera that form either determinate, indeterminate or aeschynomenoid nodules. Hence, NGR234 is a model organism to study nitrogen fixation in association with a variety of legumes. The symbiotic plasmid pSfrNGR234a carries more than 50 genes that are under the transcriptional control of NifA. To facilitate the functional analysis of NifA-regulated genes a new transposable element, Tn*EKm-PwA,* was constructed. This transposon combines the advantages of *in vitro* mutagenesis of cloned DNA fragments with a conditional read-out promoter from NGR234 (PwA) that reinitiates NifA-dependent transcription downstream of transposition sites. To test the characteristics of the new transposon, the *nifQdctA1*y4vGHIJ operon was mutated using either the Omega interposon or Tn*EKm-PwA.* The symbiotic phenotypes on various hosts as well as the transcriptional characteristics of these mutants were analysed in detail and compared with the ineffective (Fix$^-$) phenotype of strain NGRΔ*nifA*, which lacks a functional copy of *nifA*. *De novo* transcription from inserted copies of Tn*EKm-PwA* inside bacteroids was confirmed by qRT-PCR. Unexpectedly, polar mutants in *dctA1* and *nifQ* were Fix$^+$ on all of the hosts tested, indicating that none of the six genes of the *nifQ* operon of NGR234 is essential for symbiotic nitrogen fixation on plants that form nodules of either determinate or indeterminate types.

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Abbreviations: DN, determinate nodules; IDN, indeterminate nodules; Gus, β-glucuronidase; PHB, poly-β-hydroxybutyrate; p.i., post-inoculation; qRT-PCR, real-time quantitative reverse transcription polymerase chain reaction; 5′ RACE, 5′ rapid amplification of cDNA ends; UAS, upstream activating sequence.

A supplementary figure showing mapping of the start points of transcripts produced by PwA is available with the online version of this paper.

INTRODUCTION

Under conditions of limiting nitrogen, soil bacteria collectively known as rhizobia may form nitrogen-fixing symbioses with legume hosts. Reduction of atmospheric nitrogen (N_2) by rhizobia occurs predominantly inside plant cells of root or stem nodules. To protect the rhizobial nitrogenase from irreversible inactivation by traces of free oxygen, host plants have evolved a number of mechanisms,

including the formation of a cortical oxygen diffusion barrier that surrounds the infected nodule tissue, and the synthesis of leghaemoglobin, which regulates the oxygen tension within infected cells. To colonize the inner sections of nodules, rhizobia generally follow a path of infection via infection threads that cross several cell layers and are under the strict control of the host plant (Gage, 2004; Oldroyd & Downie, 2008). Therefore, successful infection of plant tissues by rhizobia requires the coordinated exchange of a series of specific molecular signals (Gibson *et al.*, 2008; Perret *et al.*, 2000). Once the tips of infection threads reach the nodule meristem, rhizobia are introduced into the host cytoplasm as infection droplets surrounded by a plasma membrane of plant origin (the peribacteroid membrane). At this stage, endocellular rhizobia differentiate into nitrogen-fixing bacteroids which, together with the peribacteroid membrane that surrounds them, are often referred to as symbiosomes (Roth *et al.*, 1988). Host plants provide rhizobia with the carbon and energy sources required to fuel symbiotic nitrogen fixation, mostly in the form of dicarboxylic acids such as malate and succinate, which are imported into bacteroids via the Dct transport system. The Dct system of rhizobia consists of a dicarboxylate carrier protein DctA and a two-component regulatory system, where DctB and DctD play the roles of sensor kinase and transcriptional activator, respectively (Yurgel & Kahn, 2004).

In legumes of the galegoid clade such as *Medicago sativa* that form nodules of indeterminate type (IDN), differentiation of *Sinorhizobium meliloti* strain 1021 into bacteroids was shown to be irreversible and controlled by the host (Mergaert *et al.*, 2006; Van de Velde *et al.*, 2010). In contrast, no terminal differentiation was observed for bacteroids isolated from hosts that form determinate nodules (DN), and senescence seemed to promote redifferentiation into free-living cells (Müller *et al.*, 2001). Although the nitrogen status within bacterial cells contributes to regulation of nitrogen-fixation genes in free-living diazotrophs, the level of free oxygen inside plant cells is the major environmental switch that triggers the expression of nitrogen-fixation genes in rhizobia (Dixon & Kahn, 2004; Fischer, 1994). In rhizobia, the oxygen-dependent regulatory cascades that activate nitrogen fixation are often mediated by FixLJ (as *in S. meliloti*: David *et al.*, 1988) but may also involve FnrN and/or RegSR (Dixon & Kahn, 2004; Lopez *et al.*, 2001). However, in both free-living and symbiotic nitrogen fixers, all *nif* genes that encode components of the nitrogen-fixing apparatus (nitrogenase and associated proteins) are under the transcriptional control of NifA (Dixon & Kahn, 2004). NifA belongs to the class of transcriptional activators known as enhancer-binding proteins (EBPs) that interact with the RNA polymerase sigma factor σ^{54} (also known as RpoN or NtrA). RpoN recognizes conserved promoter sequences (consensus 5′-TGGCAC-N_5-TTGCA/T-3′) at −12 and −24 bp relative to the transcription start site, whereas NifA binds to upstream activating sequences (UAS consensus 5′-TGT-N_{10}-AGA-3′) located 80 to 150 bp further upstream. Bioinformatic analysis using the conserved binding sites for NifA and σ^{54} identified 16 potential NifA-activated promoters in the 536 165 bp plasmid pSfrNGR234a (previously pNGR234a), the symbiotic plasmid of *Sinorhizobium fredii* strain NGR234 (Freiberg *et al.*, 1997). In addition to pSfrNGR234a, the genome of NGR234 comprises a chromosome of 3 925 702 bp that is predicted to encode 3633 ORFs, and a megaplasmid pSfrNGR234b (pNGR234b) of 2 430 033 bp with 2342 ORFs (Schmeisser *et al.*, 2009). Transcriptional analyses confirmed that the 16 predicted NifA-σ^{54}-dependent promoters of pSfrNGR234a direct the expression of more than 50 genes, many of which are clustered within a 54 kb region centred around the *nifHDK1nifENX*y4vQ operon (Perret *et al.*, 1999, 2003). In addition to 22 *nif* and *fix*/*fdx* loci, homology searches confirmed that many genes of the NifA regulon of pSfrNGR234a are conserved and NifA-regulated in other rhizobia (Masson-Boivin *et al.*, 2009; Perret *et al.*, 2003). However, their function inside bacteroids, and the contribution of many NifA-controlled loci to symbiotic nitrogen fixation, remain unknown.

First isolated from nodules of *Lablab purpureus* in 1965 by M. J. Trinick, *S. fredii* strain NGR234 became a model bacterium to study the molecular basis for broad-host-range symbioses (Pueppke & Broughton, 1999; Trinick, 1980). This strain was extensively used to characterize the molecular signals required for nodule formation on the roots of its hosts and their subsequent infection by bacteria (Perret *et al.*, 2000); however it has been less well studied with respect to its ability to fix nitrogen within plants that form either DN or IDN (Perret *et al.*, 2003; Pueppke & Broughton, 1999). Derivatives of NGR234 impaired in symbiotic nitrogen fixation include the *hemA* site-directed mutant strain NGR234ΩH1 (Stanley *et al.*, 1988), the *ntrA* (σ^{54}) deletion strain NGR234*rn1* (Stanley *et al.*, 1989; van Slooten *et al.*, 1990) and the *dctA1* mutant strain NGR*dc1* (van Slooten *et al.*, 1992), which were each unable to fix nitrogen on all plants tested (Fix$^-$). In addition, the *bdhA* mutant strain NGRPA2 was symbiotically impaired on *Leucaena leucocephala* but not on other hosts (Aneja & Charles, 2005) while a mutation in *pckA* resulted in Fix$^-$ nodules on *Vigna unguiculata* (Østerås *et al.*, 1991). Finally, there has also been structural and functional analysis of the two *nifHDK* promoters of pSfrNGR234a (Badenoch-Jones *et al.*, 1989). Thus far, none of the NifA-regulated genes found on pSfrNGR234a other than *dctA1* has been targeted for mutagenesis. To assess the function and importance of each of the bacteroid-expressed loci during symbiotic nitrogen fixation in broad-host-range symbioses, a programme of systematic mutagenesis of NifA-regulated genes was initiated. To rapidly construct non-polar mutants in genes for which transcription is NifA and σ^{54} dependent, a dedicated mutagenesis system was developed (Tn*EKm-PwA*) such that the expression of genes downstream of the transposon insertion remains NifA dependent. Here we report on the construction and testing of Tn*EKm-PwA*,

and the resulting symbiotic phenotypes of *nifA*, *nifQ*, *dctA1* and y4vG mutants of pSfrNGR234a.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* recombinants were grown at 37 °C in/on Luria–Bertani medium (Sambrook *et al.*, 1989). NGR234 and its derivative strains were grown at 27 °C in/on *Rhizobium* minimal medium supplemented with succinate (RMS) (Broughton *et al.*, 1986). Kanamycin, rifampicin and spectinomycin were added at final concentrations of 50 μg ml^{-1}. For β-glucuronidase (Gus) assays, cultures were grown as follows: cells of overnight pre-cultures of rhizobia were diluted to a density of $OD_{600}^{1\,cm}$ 0.1, induced with 2×10^{-7} M daidzein diluted in ethanol and harvested at the indicated times. In non-induced cultures, daidzein was replaced with an equivalent volume of ethanol.

Construction of Tn*EKm-PwA*. Vector pMOD2 (Epicenter Technologies) was used as the basis for Tn*EKm-PwA* assembly. First, the coding sequence (CDS) of the E-tag peptide that facilitates the detection of translation fusions with extracytoplasmic proteins was isolated from a recombinant clone of a previous phage-display experiment (Rosander *et al.*, 2003) and cloned as a short *Kpn*I–*Bam*HI fragment to generate pMOD2E-tag. Then, the promoter of the y4wAB operon (PwA) was amplified by PCR using primers PstI-P1 and HindIII-P1 (see Table 2) and NGR234 genomic DNA as template to yield one 162 bp amplicon. The product was digested with *Pst*I and *Hin*dIII and cloned into pMOD2E-tag to produce pMOD2E-PwA. The kanamycin-resistance (Km^R) Omega interposon cassette (Ω) was amplified from pHP45 (Prentki & Krisch, 1984) using primer Omega-2 and the resulting product was cloned into the unique *Xba*I site of pMOD2E-PwA to generate pMOD2EKm-PwA. On this plasmid, the complete transposable element Tn*EKm-PwA* consisting of the E-tag CDS, Km^R-resistant Ω interposon and PwA promoter was flanked on both sides by the 19 bp inverted repeats required for transposition by the EZ : TN transposase. The structure of Tn*EKm-PwA* that is detailed in Fig. 1 (top) was verified by sequencing. To prevent possible problems linked to the ColE1-based origin of replication of pMOD2, Tn*EKm-PwA* was also cloned in the R6Kγ-based vector pMOD4, which can be maintained in *E. coli* strain EC100D pir^+ background, yielding pMOD4EKm-PwA.

Mutagenesis procedures. Mutant strains deleted in *nifA* were constructed as follows. A fragment of 3083 bp which spans *nifA* and covers positions 154 653 to 157 731 of pSfrNGR234a (GenBank NC_000914.2) (Freiberg *et al.*, 1997; Schmeisser *et al.*, 2009) was amplified by PCR using primers NifA-1 and NifA-2 with pXB296 cosmid DNA (Perret *et al.*, 1991) as template. The resulting PCR product was digested by *Spe*I and cloned into a modified pBluescript KS^+ (Stratagene) in which the *Eco*RI and *Bam*HI sites were removed. Then, the 1521 bp *Bam*HI–*Bcl*I fragment internal to *nifA* was replaced with the Km^R Ω interposon to generate pCKSΔ*nifA*. To construct pNifAΩKm-1, the two *Eco*RI fragments of 489 and 525 bp that cover the 3′-end of *nifA* and half of the UAS of the *nifB* promoter were replaced with Ω Km^R. The inserts of pCKSΔ*nifA* and pNifAΩKm-1 were excised using *Spe*I, purified by agarose gel electrophoresis and subcloned into pJQ200SK (Quandt & Hynes, 1993) resulting in constructs pCJQΔ*nifA* and pJQ*nifA*ΩKm-3, respectively. Each of these two constructs was mobilized into NGR234 by triparental mating using the helper plasmid pRK2013 (Figurski & Helinski, 1979). Marker exchange was selected by plating on RMS plates containing 5 % (w/v) sucrose, yielding the *nifA*-deletion mutants NGRΔ*nifA* and NGRΔ*nifA*-1 (see Fig. 1, bottom, for sections of pSfrNGR234a that were replaced with Ω). Similar procedures were used to generate NGRΩ*dctA1* and NGRΩy4vG by inserting the Ω Km^R interposon into the *Bcl*I site of *dctA1* and the *Bam*HI site of y4vG, respectively. The NGRΔ*nifQ* mutant was obtained by replacing a 1719 bp *Xma*I fragment covering *nifQ* and most of *dctA1* with Ω (see Fig. 1, bottom). Together with their relevant properties, the various constructs used to generate the *nifA*, *nifQ*, *dctA1* and y4vG mutants of NGR234 are listed in Table 1.

Tn*EKm-PwA* mutants of NGR234 were created as follows. A 4.2 kb *Sac*I–*Sal*I fragment derived from a PCR product amplified using the specific primers Sac-dct and Sal-dct was cloned into pJQ200SK. The resulting construct pJQ-*dctA-SS*, which carries the *dctA1* to y4vI genes, was mutagenized *in vitro* as described previously (Müller, 2004) using Tn*EKm-PwA*. Mutagenized pJQ-*dctA-SS* subclones were electroporated into *E. coli* cells, and the position and orientation of Tn*EKm-PwA* in each of them was determined by DNA sequencing using primer Tn-P1 (see Fig. 1, top). A set of six independent transposition events into *dctA1*, y4vG, y4vH or y4vI were selected for gene replacement in NGR234 using a procedure similar to that described above for the Ω mutagenesis.

Construction of *gusA* fusions for transcription analysis. To monitor the expression of target loci under various conditions, the promoters of *rpsL* (P*rpsL*), *sigA* (P*sigA*), *nodA* (P*nodA*), *nifH1* (P*nifH1*) and y4wA (PwA) were cloned upstream of the promoterless β-glucuronidase gene (*gusA*) of the broad-host-range reporter system pRG960 (Van den Eede *et al.*, 1992). The selected promoters were amplified by PCR using *Pfu* DNA polymerase (Stratagene), NGR234 genomic DNA as a template, and specific primer pairs listed in Table 2. Using the appropriate restriction enzymes, amplicons of 426 (P*rpsL*), 300 (P*sigA*), 360 (P*nodA*), 499 (P*nifH1*), and 169 (PwA) nt were subsequently cloned in *Pst*I/*Bam*HI-restricted pRG960 to generate both transcriptional and translational fusions with *gusA*. Constructs were verified by sequencing using the pRG-For and pRG-Rev primers, which span the pRG960 cloning site. The resulting *gusA* fusion plasmids pXPrpsL426, pXPnodA, pXPnifH1, pYXPsigA and pYXPwA were mobilized into NGR234 by triparental mating.

Fluorometric β-glucuronidase (Gus) assays. At the indicated times, aliquots of approximately 5×10^8 cells grown in liquid cultures were harvested by centrifugation and then treated with 300 μl extraction buffer (EB) containing 50 mM sodium phosphate (pH 7.0), 0.1 % Triton X-100, 0.1 % Sarkosyl and 10 mM EDTA pH 8. Alternatively, 100 mg of nodules collected from different plants on the indicated days post-inoculation (days p.i.) were crushed together with sand in 600 μl EB. The resulting homogenates were centrifuged at 10 000 ***g*** for 10 min at 4 °C, and aliquots of the clear supernatants were used to estimate the protein concentrations by an improved colorimetric Lowry assay (DC Protein Assay; Bio-Rad). In all cases, Gus activities were quantified by incubating a maximum of 0.2 μg of each of the protein extracts with 2 mM 4-methylumbelliferyl β-D-glucuronide (4MUG; Sigma-Aldrich) diluted in EB with 10 mM 2-β-mercaptoethanol at 37 °C for up to 60 min. At various times during incubation, fluorescence was measured with a Hoefer TKO 100 mini-fluorometer calibrated according to the manufacturer's instructions. Gus activities were expressed as units of fluorescence released min^{-1} (μg protein)$^{-1}$ (Jefferson *et al.*, 1986). The results reported are the means of at least three independent experiments.

Plant assays. Surface-sterilized seeds of *Macroptilium atropurpureum*, *Leucaena leucocephala*, *Tephrosia vogelii* and *Vigna unguiculata* were dispersed on agar plates and incubated to germinate in the dark, at 27 °C. Once germinated, seedlings were planted in Magenta jars containing vermiculite (Lewin *et al.*, 1990), and watered using nitrogen-free B&D solution (Broughton & Dilworth, 1971). Two to three days after their transfer to Magenta jars, each plantlet was

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
Strains		
NGR234	Spontaneous Rif^R-derivative of the promiscuous *Sinorhizobium* strain isolated from *Lablab* by M. J. Trinick, Rif^R	Stanley *et al.* (1988)
NGRΔ*nifA*	NGR234 derivative in which the 1521 bp *Bam*HI–*Bcl*I fragment internal to *nifA* was replaced with Ω, Rif^R Km^R	This work
NGRΔ*nifA*-1	NGR234 derivative in which 489 and 525 bp *Eco*RI fragments of *nifA* were replaced with Ω, Rif^R Km^R	This work
NGRΔ*nifQ*	NGR234 derivative in which Ω replaced a 1719 bp *Xma*I fragment that covers the start of the *nifQ* operon, Rif^R Km^R	This work
NGRΩ*nifA*-4	Single-crossover intermediate mutant in which an intact copy of *nifA* is separated from *fixABCX* by pJQ*nifA*ΩKm, Rif^R Km^R	This work
NGRΩ*dctA1*	NGR234 derivative that carries Ω in the *Bcl*I site at the 5′-end of *dctA1*, Rif^R Km^R	This work
NGRΩy4vG	NGR234 derivative that carries Ω in the *Bam*HI site at the 5′-end of y4vG, Rif^R Km^R	This work
NGR117	NGR234 derivative with Tn*EKm-PwA* inserted in the 5′-end of *dctA1*, with promoter PwA oriented towards y4vG, Rif^R Km^R	This work
NGR118	NGR234 derivative with Tn*EKm-PwA* inserted in the 3′-end of y4vG, with promoter PwA oriented towards *nifQ*, Rif^R Km^R	This work
NGR(pXPrpsL426)	NGR234 transconjugant harbouring plasmid pXPrpsL426, Rif^R Sp^R	This work
NGR(pYXPsigA)	NGR234 transconjugant harbouring plasmid pYXPsigA, Rif^R Sp^R	This work
NGR(pXPnodA)	NGR234 transconjugant harbouring plasmid pXPnodA, Rif^R Sp^R	This work
NGR(pXPnifH1)	NGR234 transconjugant harbouring plasmid pXPnifH1, Rif^R Sp^R	This work
NGR(pYXPwA)	NGR234 transconjugant harbouring plasmid pYXPwA, Rif^R Sp^R	This work
NGRΔ*nifA*(pYXPwA)	NGRΔ*nifA* transconjugant harbouring plasmid pYXPwA, Rif^R Sp^R	This work
Plasmids		
pBluescript KS	ColEI-based phagemid, *lacαZ*$^+$, Ap^R	Stratagene
pJQ200SK	Versatile suicide vector, Gm^R	Quandt & Hynes (1993)
pRK2013	Tra$^+$ helper plasmid, Km^R	Figurski & Helinski (1979)
pHP45	Ω Km^R interposon	Prentki & Krisch (1984)
pRG960	Broad-host-range, *gusA* reporter system, Sp^R	Van den Eede *et al.* (1992)
pXB296	Lorist2-based cosmid clone (GenBank Z68203) carrying positions 175 928 to 205 207 of pSfrNGR234a, Km^R	Perret *et al.* (1991)
pMOD2	Transposon construction vector, ColE1, Ap^R	Epicenter Technologies
pMOD4	Transposon construction vector, R6Kγ, Ap^R	Epicenter Technologies
pMOD2E-tag	pMOD2 derivative containing the E-tag CDS, Ap^R	This work
pMOD2E-PwA	pMOD2E-tag derivative in which PwA was cloned as a 152 bp *Pst*I–*Hin*dIII fragment, Ap^R	This work
pMOD2EKm-PwA	pMOD2 derivative with the complete Tn*EKm-PwA* transposon (E-tag, Ω Km^R cassette, PwA promoter), Ap^R Km^R	This work
pMOD4EKm-PwA	pMOD4 carrying the Tn*EKm-PwA* transposon, Ap^R Km^R	This work
pCKSΔ*nifA*	pBluescript clone in which Ω replaced the 1521 bp *Bam*HI–*Bcl*I fragment internal to *nifA*, Ap^R Km^R	This work
pCJQΔ*nifA*	pJQ200SK clone containing the 3.8 kb *Spe*I fragment isolated from pCKSΔ*nifA*, Gm^R Km^R	This work
pNifAΩKm-1	pBluescript clone in which Ω replaced the 489 and 525 bp *Eco*RI fragments of *nifA*, Ap^R Km^R	This work
pJQ*nifA*ΩKm-3	pJQ200SK clone containing the 4.3 kb *Spe*I fragment isolated from pNifAΩKm-1, Gm^R Km^R	This work
pCJQΩ*dctA1*	pJQ200uc1 clone containing the 6.8 kb *Not*I fragment in which Ω is inserted in the *Bcl*I site of *dctA1*, Gm^R Km^R	This work
pCJQΔ*nifQ*	pJQ200uc1 clone containing the 5.7 kb *Not*I fragment in which Ω replaces a 1.7 kb *Xma*I fragment of the *nifQ* operon, Gm^R Km^R	This work
pJQ-*dctA-SS*	4.2 kb *Sac*I–*Sal*I fragment covering *dctA1* to y4vI and used for *in vitro* mutagenesis with Tn*EKm-PwA*, Gm^R	This work
pXJQy4vHΩKm	pJQ200SK clone containing the 6.8 kb *Not*I fragment in which Ω is inserted in the *Bam*HI site of y4vG, Gm^R Km^R	This work
pXKS*nifQ*y4vI	5.6 kb *Eco*RI–*Sal*I fragment of pXB296 cloned in pBluescript KS in which the *Bam*HI site of the polylinker was removed, Ap^R	This work

Table 1. cont.

Strain or plasmid	Relevant properties	Source or reference
pXPnifH1	Promoter of the *nifHDK1nifENX*y4vQ operon cloned as a 499 bp *Pst*I–*Bam*HI fragment in pRG960, Sp^R	This work
pXPnodA	Promoter of the *nodABCIJnolOnoeE* operon cloned as a 360 bp *Pst*I–*Bgl*II fragment in pRG960, Sp^R	This work
pXPrpsL426	Promoter of the *rpsLGfusA1tufA* operon cloned as a 426 bp *Pst*I–*Bam*HI fragment in pRG960, Sp^R	This work
pYXPsigA	Promoter of the y4wAB operon cloned as a 300 bp *Pst*I–*Bam*HI fragment in pRG960, Sp^R	This work
pYXPwA	Promoter of the y4wAB operon cloned as a 169 bp *Pst*I–*Bam*HI fragment in pRG960, Sp^R	This work

Table 2. Primers used in PCR experiments

Cleavage sites for restriction endonucleases are underlined.

Primer	Sequence (5′ to 3′)	Characteristics/use
NifA-1	CACTAGTTCGAGCGCATTTGGGC	*Spe*I site, for mutagenesis of *nifA*
NifA-2	TCACTAGTTGAATGAAGACGGC	*Spe*I site
PnifH1-for	CTCCTGCAGAAAGTTGGGCG	*Pst*I site, for cloning of P*nifH1*
PnifH1-rev	ACGGGATCCTGCCATGTTGCT	*Bam*HI site
PnodA-for	AAGCTGCAGCTCCCGCCG	*Pst*I site, for cloning of P*nodA*
PnodA-rev	CTGAGATCTCATATCCAAAGAAC	*Bgl*II site
PrpsL-for3	GAGAAGGCACTGCAGCGGC	*Pst*I site, for cloning of P*rpsL*
PrpsL-rev3	ATCGGATCCTTTACGGTAGGC	*Bam*HI site
PsigA-For1	TTCTGCAGGAAACGCTTGACG	*Pst*I site, for cloning of P*sigA*
PsigA-Rev1	TTTCTTGGATCCTTGTTGCCAT	*Bam*HI site
PwA-for1	GCGATGCATTTTTAGCCGC	*Nsi*I site, for cloning of PwA
PwA-rev1	TGAGATCTAGACATCCTCCGC	*Bgl*II site
PstI-P1	CTGCAGTTTTAGCCGCCAGGCTG	*Pst*I site
HindIII-P1	AAGCTTCTCCGCACAATTCTCGTCG	*Hin*dIII site
Omega-2	GACTCGAGATCTAGAATTCCCGGGGATCCGGTG	*Xba*I site
Sac-dct	GAGAGGCTTGAGCTCAAGCG	*Sac*I site
Tn-P1	CGTGGCACGTATCTTGCTCTGCTG	To sequence mutagenized plasmids
Sal-dct	CCACAAGCGTCGACCCGG	*Sal*I site
pRG-For	GTGCTGCAATGATACCGCGAG	Upstream of pRG960 cloning site
pRG-Rev	GAATGCCCACAGGCCGTCGAG	Downstream of pRG960 cloning site
y4wA-RP1	AATTGCAGTGCGACCATACA	For mapping +1 of y4wAB transcript
y4wA-RP2	CATAGGACAAATGCACAGATA	
y4wA-RP3	GATACGCGAACACACTCCAT	
y4vG-RP1	GCTGGTCTCTCGCCCGTG	For mapping PwA-dependent +1 in y4vG
y4vG-RP2	CCGTGTTATCACCCAGGTAC	
y4vG-RP3	GTACCTCGTCCATCGCGCG	
16S-RT-For	AGCCACATTGGGACTGAGAC	For quantification of 16S rRNA
16S-RT-Rev	ACCCTAGGGCCTTCATCACT	
rpsL-RT-For	GCTTCGAAGTGATCGGCTAC	For quantification of *rpsL* transcript
rpsL-RT-Rev	ACACCCTGCGTATCGAGAAC	
nifQ-RT-For	CAAAACATGCGGTGGAAGAAG	For quantification of *nifQ* transcript
nifQ-RT-Rev	CAAAGCAGCTCTCCAATTCC	
y4vG-RT-For	CCCCAACACTATCGATCTGG	For quantification of y4vG transcript
y4vG-RT-Rev	CGTAGGCCAATGACGATTTC	
y4vI-RT-For	GATTCTCGCGGATTGAAGTG	For quantification of y4vI transcript
y4vI-RT-Rev	GCTAGAGCGCGTTGAAAATC	

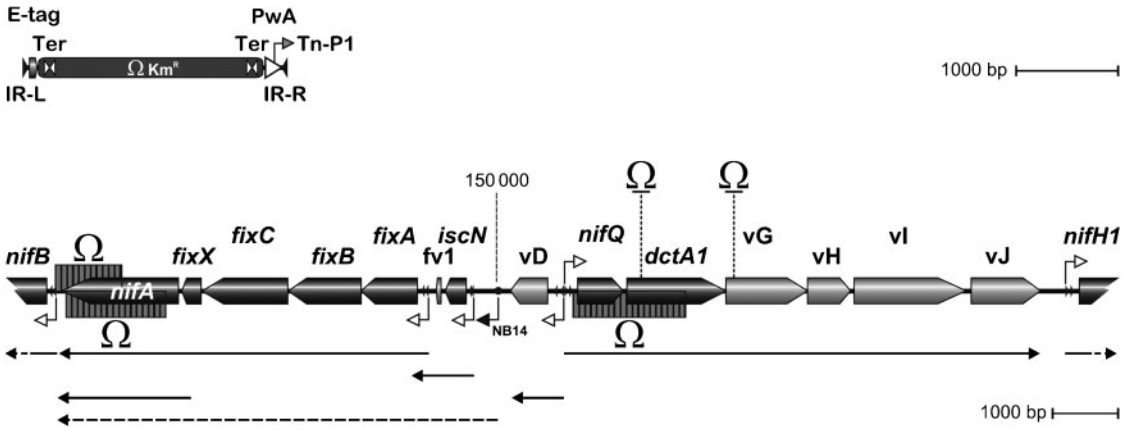

Fig. 1. Structure of the Tn*EKm-PwA* transposon and genetic organization of a section of pSfrNGR234a. Top, structure and major characteristics of Tn*EKm-PwA* with its terminal inverted repeats IR-L and IR-R (black triangles), the NifA-σ^{54}-dependent promoter PwA oriented according to the direction of transcription (white triangle), the transcription terminators Ter (facing white triangles) at both ends of the kanamycin-resistant Omega interposon (Ω Km^R), and the sequencing primer Tn-P1, drawn as an arrow. Bottom, positions 157 501 to 140 500 of pSfrNGR234a (NC_000914.2) with the predicted ORFs drawn as arrows oriented according to the direction of transcription. Positions and orientations of NifA-σ^{54}-dependent promoters are marked with white arrows, with the corresponding predicted transcripts shown below the genetic map. The flavonoid-dependent transcript controlled by *nod*-box NB14 is represented as a dashed line. The positions of the Omega interposon (Ω) within mutant strains in *dctA1* (NGRΩ*dctA1*) and y4vG (NGRΩy4vG) are shown, while sections it replaces in NGRΔ*nifA,* NGRΔ*nifA*-1 and NGRΔ*nifQ* are hatched.

inoculated with 2×10^8 bacteria. All plants were grown 6 to 8 weeks post-inoculation at a day temperature of 27 °C, a night temperature of 20 °C and a light phase of 12 h. Nodules were prepared for electron microscopy as described by Golinowski *et al.* (1987).

RNA preparations. At the indicated times, bacteria from liquid cultures were isolated by centrifugation (10 min, 8000 ***g*** at 4 °C) and the cell pellets immediately frozen in liquid nitrogen and stored at −60 °C. Unless specified otherwise, root nodules of *V. unguiculata* were collected 42 days p.i., frozen in liquid nitrogen, and stored at −60 °C until further use. For the subsequent isolation of bacterial RNA, 1 g of frozen nodules was ground in 20 ml ice-cold MES buffer (350 mM mannitol, 25 mM MES pH 6.0, 3 mM $MgSO_4.7H_2O$, pH 7) using a mortar and pestle. The homogenate was filtered through four layers of Miracloth and centrifuged at 4 °C for 3 min at 1000 ***g*** to remove contaminating plant materials. Bacteroids in the supernatant were sedimented for 12 min at 8000 ***g*** and 4 °C. To isolate total RNA, approximately 5×10^9 free-living cells or bacteroids were resuspended in 5 ml of a prewarmed 50/50 volume of pH 4.3 phenol (Sigma-Aldrich) and NETS 2× buffer (400 mM NaCl, 1 mM EDTA, 20 mM Tris/HCl pH 8.0, 1 % SDS), and incubated for 30 s in boiling water. Following a centrifugation of 5 min at 16 000 ***g*** and 4 °C, the aqueous phase was extracted several times with an equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1, by vol.). Nucleic acids were precipitated from the supernatant with 1/3 vol. 8 M LiCl and 2 vols ethanol. Contaminating genomic DNA was removed by two consecutive DNase I treatments on columns (Qiagen). Total RNA samples were examined on agarose gel for possible degradation, and their concentration and purity determined by spectrophotometry.

cDNA synthesis and real-time quantitative reverse transcription PCR (qRT-PCR). Reverse transcription was carried out using 1 μg of total RNA in a final volume of 20 μl, 1 μM of random hexamers and the iScript Select cDNA Synthesis kit (Bio-Rad). Expression of target loci was measured by rqRT-PCR with an iCycler iQ instrument. Primers were designed using the online Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to generate amplicons of approximately 100 to 150 bp (see Table 2 for primer sequences). Each amplification was carried out in duplicate, in a 20 μl reaction mix containing 5 μl of 10-fold diluted cDNA template, 10 μl SYBR Green Supermix (Bio-Rad) and a 500 nM concentration of each primer. After an initial activation step of 3 min at 95 °C, the thermocycler was programmed for 40 cycles of 30 s at 95 °C and 1 min at 60 °C.

Mapping of transcription start sites. The transcription start site (+1) of the y4wAB operon was mapped by 5′ rapid amplification of cDNA ends PCR (5′ RACE-PCR). Briefly, the first cDNA strand was synthesized from 1 μg total RNA of bacteroids using 625 nM primer y4wA-RP1, the AMV reverse transcriptase and the deoxynucleotide mixture provided with the 3′/5′ RACE kit (Roche Diagnostics). Following the synthesis of a 3′-poly(A)$^+$ tail on the first cDNA strand, a first PCR was carried out with the oligo dT-anchor primer and the specific y4wA-RP2 oligonucleotide. The resulting product was diluted 20-fold and used for nested PCR with the anchor primer and y4wA-RP3. Both PCR products were purified, and their nucleotide sequences determined with primer y4wA-RP3. The PwA-dependent +1 of y4vG in the Tn*EKm-PwA*-derived mutant strain NGR117 was mapped using primers y4vG-RP1, y4vG-RP2 and y4vG-RP3 (Table 2).

RESULTS

Symbiotic phenotypes of the *nifA*-deletion mutants

As in *S. meliloti* strain 1021, *nifA* of pSfrNGR234a is found downstream of the *fixABCX* and upstream of the *nifBfdxNnifZfixU* operons (see Fig. 1, bottom). To test its

role in the regulation of nitrogen fixation in NGR234, mutant strains deleted in *nifA* were constructed (NGRΔ*nifA* and NGRΔ*nifA*-1). On all plants tested, both NGRΔ*nifA* and NGRΔ*nifA*-1 elicited the formation of numerous but small non-fixing nodules (Nod^+/Fix^- phenotype). In the absence of nitrate, the growth of host plants inoculated with NGRΔ*nifA* or NGRΔ*nifA*-1 appeared impaired when compared to non-inoculated controls, presumably because a significant portion of the seed's internal resources was allocated to the development of non-fixing nodules. Although not unexpected given the inability of rhizobial *nifA* mutants to perform symbiotic nitrogen fixation, this phenotype became our reference Fix^- phenotype. These results also confirmed that in the parent NGR234 strain no other RpoN-dependent transcriptional regulator was capable of complementing the absence of *nifA* for symbiotic nitrogen fixation (Labes *et al.*, 1993).

Comparative cytological analysis of nodules induced on *V. unguiculata* confirmed that both NGRΔ*nifA*-1 and the parent strain are equally capable of colonizing nodule cells (Fig. 2). This indicates that the Fix^- phenotype of NGRΔ*nifA*-1 results from its inability to perform nitrogen fixation rather than a failure to infect the developing nodules. In fact, at 28 days p.i. nodules formed by NGRΔ*nifA*-1 or NGR234 showed a similar distribution of infected and non-infected zones. Thus, the infection process and the release of bacteria from infection threads are not prematurely terminated in *V. unguiculata* when *nifA* is missing. However, detailed structures of infected cells differed considerably. Bacteroids of NGRΔ*nifA*-1 accumulated higher levels of poly-β-hydroxybutyrate (PHB), and the NGRΔ*nifA*-1 symbiosomes often fused, while the cytoplasm of host cells showed signs of degradation (see Fig. 2c, bottom), features that were either absent or less pronounced in nodules infected by NGR234.

Reduced symbiotic nitrogen fixation by the *cis*-merodiploid strain NGRΩ*nifA*-4

During the construction of the NGRΔ*nifA*-1 deletion mutant, a number of intermediate strains were produced which provided additional insight into the regulation of *nifA*. For example NGRΩ*nifA*-4 is a *cis*-merodiploid strain in which the integration of the pJQ*nifA*ΩKm construct resulted in an intact copy of *nifA* that was isolated from the *fixA* promoter but preceded by the 52 bp *fixX*–*nifA* intergenic region and the 3′-end of *fixX*. Interestingly, NGRΩ*nifA*-4 fixed nitrogen inside nodules of *V. unguiculata*, albeit at a much lower level than the parent strain. At 36 days p.i., plants inoculated with NGRΩ*nifA*-4 had a shoot dry weight reduced by about 75 % when compared to plants inoculated with NGR234 (data not shown). The significantly smaller nodules induced by NGRΩ*nifA*-4 contained infected plant cells with an internal structure

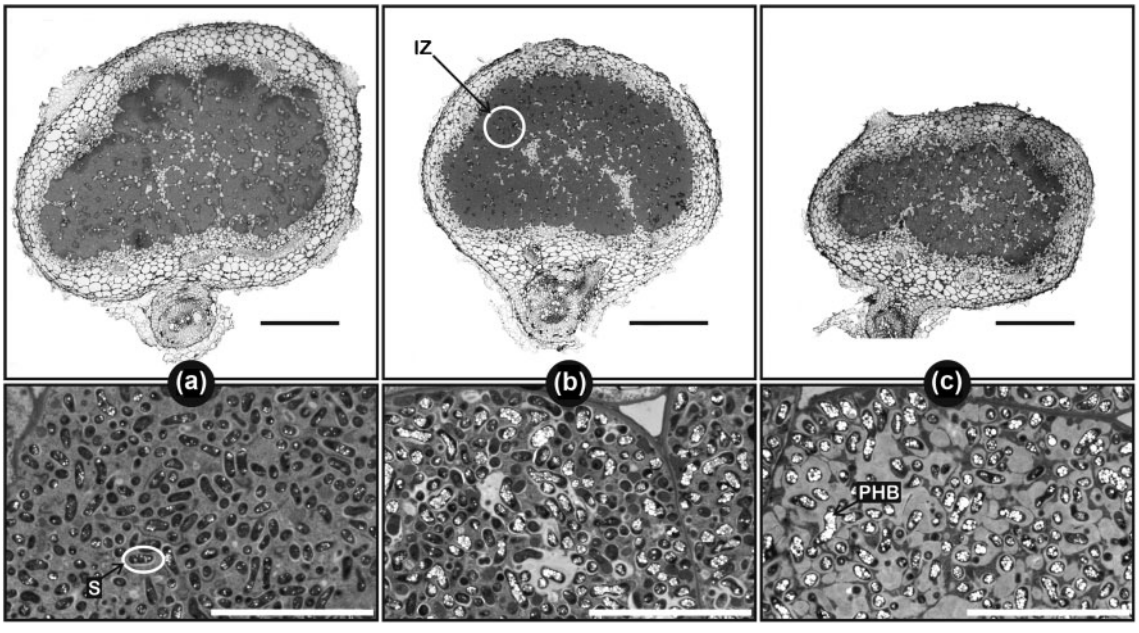

Fig. 2. Electron micrographs of sections of *V. unguiculata* nodules 28 days p.i. with strains NGR234 (a), NGRΩ*nifA*-4 (b) and NGRΔ*nifA*-1 (c). Top, cross-sections of nodules with the distribution of infected and uninfected cortical cells seen at low magnification (scale bars, 500 μm). Bottom, corresponding sections of nodules seen at a higher magnification and showing the details of plant cells containing bacteroids (scale bars, 10 μm). Granules of PHB are seen as electron-transparent droplets in the cytoplasm of bacteroids. IZ, infected zone; S, symbiosome formed by an isolated bacteroid surrounded by a peribacteroid membrane.

that was intermediate between those of cells infected with the parent or the NGRΔ*nifA*-1 strain: the host cytosol showed some signs of degradation, and bacteroids that accumulated PHB were occasionally seen in multiple-unit symbiosomes (see Fig. 2b, bottom). A possible explanation for this phenotype is that the 178 bp sequence immediately upstream of the intact *nifA* copy in NGRΩ*nifA*-4 was sufficient to sustain the synthesis of low levels of functional NifA inside bacteroids. In this context, the enhanced expression of *nifA* from P*fixA* would be essential to achieve optimal levels of nitrogen fixation in bacteroids of NGR234, and prevent the infected plant cells from showing premature signs of senescence.

Characterization of the NifA-σ^{54}-dependent promoter of the y4wAB operon

Transcription analysis of genes encoded by pSfrNGR234a showed that the y4wAB operon was strongly expressed inside nitrogen-fixing bacteroids isolated from nodules of *V. unguiculata* and *Cajanus cajan* (Perret *et al.*, 1999, 2003). *In silico* analysis of the region upstream of y4wA showed the presence of a conserved binding site for σ^{54} (5′-TGGCACgtatcTTGCT-3′) 46 bp upstream of the predicted ATG start codon and a potential UAS for NifA (5′-TGTtcgacatacgACA-3′) a further 110 bp upstream (Dombrecht *et al.*, 2002; Perret *et al.*, 2003). To follow its activity in bacteroids and free-living cells of NGR234, the promoter of y4wA (PwA) was cloned as a 169 bp *Nsi*I–*Bgl*II fragment into *Pst*I/*Bam*HI-restricted pRG960 to generate a transcriptional fusion called pYXPwA. pRG960 is a spectinomycin-resistant (Sp^R) broad-host-range vector that carries a promoterless gene for β-glucuronidase (Van den Eede *et al.*, 1992). Stably maintained even in the absence of antibiotics and found in low copy number in NGR234, pRG960 is particularly suited to measure transcription activities of cloned promoters in bacteroids. In order to compare the expression patterns of PwA with those of loci from distinct functional classes, the promoters of *rpsL* (P*rpsL*), *sigA* (P*sigA*), *nodA* (P*nodA*) and *nifH1* (P*nifH1*) were also cloned into pRG960. The gene *rpsL* codes for the 30S ribosomal subunit protein S12 and is the first gene of the well conserved '*str*' chromosomal operon, which includes the essential housekeeping genes *rpsG*, *fusA1* and *tufA*. The major sigma subunit SigA (RpoD) of NGR234 is encoded by the chromosomal gene NGR_c22670 (Schmeisser *et al.*, 2009). P*nodA* comprises the NodD1-dependent and *nod*-box NB8 regulatory element that controls the flavonoid-inducible and symbiotically essential *nodABCIJnolOnoeI* operon of pSfrNGR234a (Kobayashi *et al.*, 2004; Perret *et al.*, 1999). P*nifH1* is the promoter region of the *nifHDK1nifENX*y4vQ transcription unit of pSfrNGR234a. The symbiotic plasmid also carries a second functional *nifHDK* copy that is embedded in the *nifHDK2*y4xDEF operon (Badenoch-Jones *et al.*, 1989; Freiberg *et al.*, 1997). The resulting fusion plasmids, pXPrpsL426, pYXPsigA, pXPnodA and pXPnifH1, were used as reference constructs to compare the respective activities of promoters that direct housekeeping, flavonoid-inducible or nitrogen-fixation functions. The activities of the cloned P*nifH1*, P*nodA*, P*rpsL*, P*sigA* and PwA in free-living cultures grown in the presence or absence of daidzein, or inside 38 days p.i. nodules of *V. unguiculata* are reported in Table 3. These results clearly indicate that in free-living conditions, PwA was silent in rapidly dividing cells ($OD_{600}^{1\,cm}$ 0.3), in contrast to the P*rpsL* or P*sigA* promoters. While the presence of 2×10^{-7} M daidzein in the growth medium activated the *nod*-box-controlled promoter of *nodA* to a level comparable to that of P*sigA* and P*rpsL*, daidzein had no stimulating effect on PwA. Once bacteria reached nodule cells and began fixing nitrogen, PwA became 10 times more active than P*rpsL* and was found to be even more active than the reference P*nifH1* promoter. To confirm that the activity of PwA measured inside nitrogen-fixing nodules was dependent upon σ^{54} as was predicted by bioinformatic

Table 3. Activities of selected promoters of NGR234 cloned in pRG960

Free-living cells were collected 6 h post-induction when cultures had reached an OD_{600} of 0.3. At 38 days p.i., all nodules were Fix^+. Gus activities are expressed as units of fluorescence released min^{-1} (μg protein)$^{-1}$ (Jefferson *et al.*, 1986). The results are reported as the means of at least three independent experiments, with the standard deviations shown in parentheses.

Promoter	Activities of promoters cloned in pRG960 in		
	Free-living cells 6 h post-induction with		Bacteroids of 38 days p.i. nodules
	Ethanol	Daidzein	
P*rpsL*	144.2 (±3.8)	156.45 (±3.9)	9.8 (±0.6)
P*sigA*	108.0 (±4.6)	111.1 (±9.9)	3.8 (±0.4)
P*nodA*	1.4 (±0.3)	107.6 (±5.0)	3.0 (±0.4)
P*nifH1*	1.6 (±0.3)	1.6 (±0.1)	70.1 (±8.3)
PwA	0.9 (±0.1)	1.0 (±0.1)	102.2 (±1.7)

analyses, the transcription start of y4wA was determined by 5′ RACE-PCR. Using NGR234 bacterial RNA isolated from 42 days p.i. nodules of *V. unguiculata*, the +1 site of y4wAB transcripts was mapped 10 nt downstream of the predicted binding site for RpoN (see Supplementary Fig. S1, available with the online version of this paper). As only basal levels of Gus activity were detected inside nodules formed by NGRΔ*nifA*(pYXPwA) transconjugants, a functional NifA was required for full PwA activity inside bacteroids (data not shown). Together these results confirmed that the activities of P*nifH* and PwA were limited to the endosymbiotic life-style of NGR234, and that PwA recruits NifA and σ^{54} for its activity in nitrogen-fixing bacteroids.

Tn*EKm-PwA*, a minitransposon with a conditional read-out promoter

Designed to facilitate the functional analysis of rhizobial loci expressed inside nitrogen-fixing nodules, Tn*EKm-PwA* includes the following features: (i) the Ω interposon that confers kanamycin-resistance (Km^R) to candidate recombinant strains and interrupts transcription at the transposon insertion site (TIS), and (ii) the PwA promoter that restores NifA- and σ^{54}-dependent expression immediately downstream of the TIS for genes which are properly oriented. Although not tested herein, Tn*EKm-PwA* also allows for the E-tag-mediated detection of proteins for in-frame fusions with ORFs upstream of the TIS (Rosander *et al.*, 2003). In addition, the 19 bp inverted repeats that flank Tn*EKm-PwA* allow for *in vitro* mutagenesis of cloned fragments, prior to mobilization of the selected recombinant molecules into a host rhizobial strain. The small size of PwA and the prerequisite of transposase EZ : TN for Tn*EKm-PwA* transposition contribute to maximizing the stability of transposon insertions once mutations are introduced into the host genome. Although Tn*EKm-PwA* interrupts transcription of any gene or operon at the site of its insertion, when properly oriented it creates non-polar mutations in NifA-controlled loci as a strong, bacteroid-specific and PwA-dependent expression is restored downstream of the TIS.

Functional analysis of the *nifQdctA1*y4vGHIJ operon

To verify the characteristics of Tn*Km-PwA*, the *nifQdctA1*y4vGHIJ operon of pSfrNGR234a was selected as a target for mutagenesis. This operon of 7060 bp is under the control of NifA (P*nifQ*) and is strongly expressed inside nodules (Freiberg *et al.*, 1997; Perret *et al.*, 1999). In *Azotobacter vinelandii*, *Klebsiella pneumoniae* and *Rhodobacter capsulatus*, NifQ was shown to be essential for diazotrophic growth, although an excess of molybdate in the growth medium attenuated the *nifQ* phenotype (Imperial *et al.*, 1984; Moreno-Vivian *et al.*, 1989; Rodríguez-Quiñones *et al.*, 1993). More recently, *in vitro* assays showed that NifQ donates molybdenum to a putative NifEN/NifH complex during synthesis of the nitrogenase FeMo-cofactor (Hernandez *et al.*, 2008). Of the two copies of *dctA* found in NGR234, *dctA1* is encoded by pSfrNGR234a (van Slooten *et al.*, 1992), while *dctA2* is carried by the 2 430 033 bp megaplasmid pSfrNGR234b, and together with *dctB* and *dctD* forms the conserved Dct locus of NGR234 (Schmeisser *et al.*, 2009). Initially, *dctA1* was reported to be non-essential for the growth of NGR234 in free-living conditions but required for symbiotic nitrogen fixation with *M. atropurpureum*, *L. leucocephala* and *V. unguiculata* (van Slooten *et al.*, 1992). To assess the role in symbiosis of genes downstream of *dctA1*, the Km^R Ω interposon was first inserted into the *Bam*HI site at the 3′-end of y4vG to generate the polar mutant NGRΩy4vG (Fig. 1, bottom). Although the expression of y4vG, y4vH, y4vI and y4vJ inside bacteroids was abolished, the symbiotic proficiency of NGRΩy4vG 42 days p.i. on *V. unguiculata* seemed unaffected when compared to the wild-type strain (data not shown). This indicated that none of the four genes downstream of and co-transcribed with *dctA1* was essential for nitrogen fixation. Thus, the *nifQdctA1*y4vGHIJ transcription unit, which combined genes either dispensable (e.g. y4vGHIJ) or presumably essential (*dctA1*) for nitrogen fixation, seemed an appropriate target to test the genetic characteristics of the new Tn*EKm-PwA* transposon.

Accordingly, a number of additional mutants in the *nifQdctA1*y4vGHIJ operon were generated via the *in vitro* transposition of Tn*EKm-PwA* into the 4243 bp *Sac*I–*Sal*I fragment of pJQ-*dctA-SS* and the subsequent mobilization of selected constructs into NGR234 (for details see Methods). The respective positions and orientations of Tn*EKm-PwA* in derivative strains NGR008, NGR107, NGR110, NGR113, NGR117 and NGR118 are shown in Fig. 3(a). Two mutant strains, NGR117 and NGR118, were selected for further analysis. In NGR117 the transposon is inserted at the 3′-end of *dctA1* and its embedded PwA promoter is oriented towards y4vG and the end of the operon (see Fig. 3a). In NGR118 the PwA promoter of the transposon is inserted at the 5′-end of y4vG but is oriented toward the 5′-end of *nifQ* and the beginning of the operon. As shown in Fig. 3(a), transcription of y4vGHIJ was expected to be restored in a PwA-dependent manner inside nitrogen-fixing bacteroids of NGR117. In contrast, y4vGHIJ was likely to remain silent in strain NGR118 since PwA of Tn*EKm-PwA* was directed towards *nifQ*. To confirm that the activity of the introduced PwA promoter was not compromised by the inverted repeats that border the transposon, the expression of reference genes located upstream and downstream of the transposition sites was monitored inside bacteroids of NGR117, NGR118 and NGR234 (see Fig. 3b). To do so, *V. unguiculata* plants were inoculated with NGR117, NGR118 or NGR234. At 42 days p.i., all roots carried nitrogen-fixing nodules and the plants appeared healthy, indicating that NGR117 and NGR118 were Fix^+ on *V. unguiculata* (data not shown). Total RNA was extracted from these nodules and used as template to follow the expression of *rpsL*, *nifQ*, y4vG and y4vI by

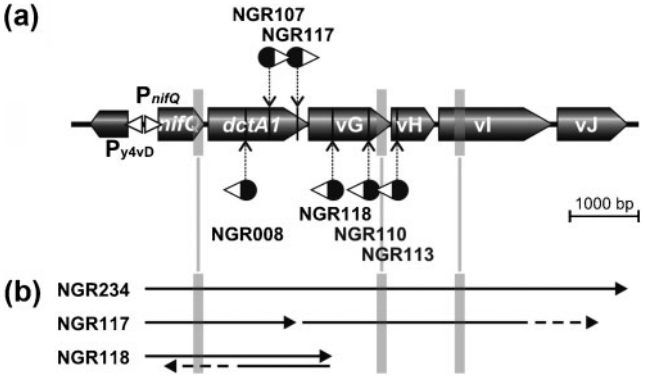

Fig. 3. Position and orientation of the Tn*EKm-PwA* transposon in the corresponding mutant strains of NGR234. (a) Detailed structure of the *nifQdctA1*y4vGHIJ operon, with inserted Tn*EKm-PwA* elements depicted as black and white cones that point towards the gene downstream of the embedded PwA promoter. (b) Size and orientation of transcripts predicted in the NGR234, NGR117 and NGR118 genetic backgrounds. Transcripts of unknown lengths that originate within Tn*Km-PwA* are represented as dashed arrows. The positions of the amplicons targeted with qRT-PCR are shaded in grey.

qRT-PCR using 16S rRNA to normalize the data. As shown in Table 4, the expression of the reference locus *rpsL* was only marginally affected in both mutant strains. In contrast, the expression of y4vG and y4vI, which remained close to wild-type levels in NGR117, was abolished in strain NGR118. These results demonstrate that (i) the transcription of y4vGHIJ is restored downstream of the site of Tn*EKm-PwA* insertion in NGR117, and (ii) the Ω interposon of Tn*EKm-PwA* efficiently blocks transcription of the loci downstream of the insertion site (i.e. y4vGHIJ in NGR118). Interestingly, the relative expression of *nifQ* was 1.7-fold higher in NGR118 than in NGR234 (Table 4). The activity of PwA of the transposon in NGR118 is the most likely explanation for this apparent increase in *nifQ* expression since both of the P*nifQ* and PwA complementary transcripts were converted into template cDNA for qRT-PCR during reverse transcription with random hexamers (see Fig. 3). To verify that the *de novo* transcription activity that was detected inside bacteroids of NGR117 downstream of the transposon was truly NifA- and σ^{54}-dependent, the transcription start of y4vG was mapped using primers y4vG-RP1, y4vG-RP2, y4vG-RP3. The 5′ RACE-PCR confirmed that the y4vG transcript initiated in NGR117 began 10 bp downstream of the conserved binding site for RpoN found within Tn*EKm-PwA*, as was the case for the y4wAB transcript found in the parent strain (see Supplementary Fig. S1).

Table 4. Relative expression of target genes in bacteroids of the Tn*EKm-PwA*-mutant strains NGR117 or NGR118 compared to the wild-type NGR234

Total RNA was purified from 42 days p.i. nodules. Positions of the amplicons selected to follow the expression of *nifQ*, y4vG and y4vI in the genetic background of strain NGR234, NGR117 or NGR118 are shown in Fig. 3(b). *rpsL* is carried on the chromosome. Results were normalized using 16S rRNA, and are reported as the means of four independent qRT-PCRs, using as template cDNAs made from two independent RNA preparations and nodule batches. For each value, the standard deviation is shown in parentheses.

Strain	Relative expression compared to wild-type for			
	rpsL	*nifQ*	y4vG	y4vI
NGR117	0.91 (±0.25)	1.06 (±0.06)	0.8 (±0.10)	0.72 (±0.17)
NGR118	0.96 (±0.23)	1.75 (±0.42)	0.01 (±0.00)	0.04 (±0.01)

As the original NGR*dc1* strain constructed by van Slooten *et al.* (1992) could not be obtained, a new NGRΩ*dctA1* mutant strain was constructed by inserting the Km^R Ω cassette into the *Bcl*I site located 217 bp downstream of the *dctA1* start codon. Unexpectedly, NGRΩ*dctA1* formed Fix^+ nodules on *L. leucocephala* (IDN), *M. atropurpureum* (DN), *Tephrosia vogelii* (IDN) and *V. unguiculata* (DN) (Table 5). The genotype of bacteria inoculated on plants and rescued from nitrogen-fixing nodules was confirmed by Southern blots as well as PCR. Furthermore, two distinct and independent recombinant strains of NGRΩ*dctA1* showed symbiotic performances similar to NGR234 on *M. atropurpureum*, *T. vogelii* and *V. unguiculata* (data not shown). Only plants of *L. leucocephala* showed growth that was slightly impaired (by about 13 %) 56 days p.i. with NGRΩ*dctA1* (see Table 5). To exclude that a deletion within *nifQ* was responsible for the Fix^- phenotype reported previously for NGR*dc1* (van Slooten *et al.*, 1992), a polar mutant called NGRΔ*nifQ*, in which the whole *nifQ* and most of *dctA1* sequences were replaced with the Ω interposon, was constructed and tested on host plants. As shown in Table 5, NGRΔ*nifQ* formed Fix^+ nodules on *L. leucocephala* (IDN), and *V. unguiculata* (DN). Notably, the shoot dry weight of *L. leucocephala* plants was reduced by 30 % when inoculated with NGRΔ*nifQ*, indicating that NifQ contributes significantly to the fine-tuning of the symbiosis with some legume hosts. Although we cannot explain the discrepancy with the Fix^- phenotype published earlier for NGR*dc1* (van Slooten *et al.*, 1992), our results demonstrate that *dctA1* and *nifQ* of pSfrNGR234a are not essential for nitrogen fixation in association with all of the NGR234 hosts that were tested.

DISCUSSION

As in other rhizobia, NifA of *S. fredii* NGR234 plays a key role in symbiotic nitrogen fixation. Unlike *Mesorhizobium loti* strains MAFF303099 and R7A, which carry two copies of *nifA* within their respective symbiosis island (Kaneko *et al.*, 2000; Sullivan *et al.*, 2002), NGR234 has a single *nifA* gene found on pSfrNGR234a (Freiberg *et al.*, 1997; Schmeisser *et al.*, 2009). As anticipated, the inactivation

Table 5. Symbiotic performance of NGR234 and the NGRΩ*dctA1* and NGRΔ*nifQ* polar mutants

Nodulation tests were performed in Magenta jars. The symbiotic phenotype of each inoculant is reported as the mean nodule number (NN), nodule fresh weight (NFW), and shoot dry weight (SDW) per inoculated plant, 42 to 56 days p.i. Results are the means of at least 14 plants per inoculant, with the standard deviations shown in parentheses. All nodules fixed nitrogen (Fix^+). NT, Not tested. Mean SDW of non-inoculated control plants was 65.5 (±12.5), 25.3 (±7.3), 149.0 (±47.6) and 113.3 (±4.4) mg for *L. leucocephala*, *M. atropurpureum*, *T. vogelii* and *V. unguiculata*, respectively.

Legume tested	Inoculant	NN	NFW (mg)	SDW (mg)
L. leucocephala (56 days p.i.)	NGR234	12.3 (±4. 8)	192.7 (±63.9)	393.8 (±86.4)
	NGRΩ*dctA1*	13.6 (±4.2)	177.9 (±53.6)	342.9 (±88.6)
	NGRΔ*nifQ*	17.2 (±8.0)	151.4 (±23.4)*	282.5 (±64.2)*
M. atropurpureum (56 days p.i.)	NGR234	48.5 (±20.4)	387.0 (±89.5)	662.0 (±173.9)
	NGRΩ*dctA1*	46.7 (±14.1)	437.9 (±212.9)	653.2 (±367.3)
	NGRΔ*nifQ*	NT	NT	NT
T. vogelii (42 days p.i.)	NGR234	9.9 (±3.8)	330.6 (±138.1)	331.1 (±104.9)
	NGRΩ*dctA1*	10.4 (±3.1)	304.6 (±98.9)	367.6 (±110.2)
	NGRΔ*nifQ*	NT	NT	NT
V. unguiculata (42 days p.i.)	NGR234	66.6 (±22.9)	691.8 (±176.4)	1130.4 (±335.1)
	NGRΩ*dctA1*	71.2 (±12.2)	667.4 (±140.2)	954.1 (±211.5)
	NGRΔ*nifQ*	72.4 (±23.5)	753.0 (±239.9)	1148.6 (±380.5)

*Values obtained with strains NGRΩ*dctA1* or NGRΔ*nifQ* that are significantly different from that of NGR234 at the level $\alpha=5\,\%$.

of *nifA* renders NGR234 Fix^- on all of the hosts tested. Previous studies suggested that *nifA* mutants not only failed to fix nitrogen, but also suffered premature bacteroid degradation and dramatic disturbances in the development of infected nodule cells (Fischer *et al.*, 1986; Hirsch & Smith, 1987). Similar conclusions could be drawn from the electron micrographs of sections of *V. unguiculata* nodules inoculated with NGRΔ*nifA*-1, and to a lesser extent with NGRΩ*nifA*-4 (see Fig. 2). In addition to the apparent dissolution of the host cytoplasm as well as a premature senescence of symbiosomes, bacteroids of NGRΔ*nifA*-1 accumulated higher levels of PHB than the parent strain. Interestingly, similar observations were made with the NGRΩ*nodD2* mutant, in which repression of nodulation genes does not occur after the initial induction with flavonoids, and which provokes infected but Fix^- nodules on *V. unguiculata* (Fellay *et al.*, 1998). Bacteroids of NGR234 were seen to produce PHB granules in DN of *V. unguiculata* (van Slooten *et al.*, 1990), *Pachyrhizus tuberosus* (Viprey *et al.*, 1998), and *M. atropurpureum* (Relić *et al.*, 1993). PHB is generally not accumulated by narrow-host-range rhizobia such as *S. meliloti* or *R. leguminosarum* that associate with plants that form nodules of indeterminate type (Trainer & Charles, 2006). However, electron micrographs confirmed the presence of PHB in bacteroids of NGR234 found in IDN of *Crotalaria juncea* (Marie *et al.*, 2003), and *T. vogelii* (V. Viprey, A. Del Greco, W. Golinowski, W. J. Broughton & X. Perret, unpublished). This indicates that PHB accumulation during symbiosis does not solely depend upon the physiology of the nodule formed by the host (Trainer & Charles, 2006), but is also dependent on the metabolism of its microsymbionts. In this respect, the levels of PHB accumulation in NGRΩ*nifA*-4 bacteroids were intermediate between those observed for endosymbiotic cells of the wild-type strain and its *nifA*-deletion derivative (Fig. 2). This is consistent with the intermediate level of nitrogen fixation by this mutant and supports the concept that, in conditions of lower nitrogen fixation and/or perturbed carbon metabolism, bacteroids tend to store more PHB. The presence of numerous PHB granules inside bacteroids and the increased number of nodules on the roots of plants inoculated with the *nifA*-deletion mutant indicate that host plants continue to provide abundant dicarboxylates to non-functional nodules even though they compensate for the reduced symbiotic performance of mutant inoculant strains by developing more nodules.

Results obtained with NGRΩ*nifA*-4 also showed that levels of *nifA* expression correlate with the effectiveness of the symbiosis and that, as in *S. meliloti* (Kim *et al.*, 1986), the enhanced expression from the NifA-σ^{54}-dependent promoter of the *fixABCXnifA* operon (P*fixA*) fulfils this role inside bacteroids. What triggers the initial expression of *nifA* in NGR234 is unknown, however. Sequence analysis confirmed that the NGR234 genome does not encode conserved FixLJ proteins (Schmeisser *et al.*, 2009). As a polar mutation within the *nod*-box-regulated gene y4vC did not significantly impair symbiotic nitrogen fixation (A. Del Greco & X. Perret, unpublished), priming of the NifA-regulatory cascade is most probably independent of NodD1 and flavonoids, and possibly controlled by a cryptic promoter located in the short *fixX–nifA* intergenic region. Current results obtained with the set of reference Gus-reporter constructs confirmed that, even in the presence of inducing flavonoids, NifA-σ^{54}-dependent promoters such as PwA or P*nifH1* are silent in free-living cells (Table 3).

Interestingly, the activity of P*nodA* inside nitrogen-fixing bacteroids isolated from 38 days p.i. nodules was close to basal levels. This indicates that the repression by NodD2 of the flavonoid-inducible regulatory cascade in control of loci preceded by *nod*-boxes on pSfrNGR234a (Fellay *et al.*, 1998; Kobayashi *et al.*, 2004) persists inside nodule cells. In contrast to P*nodA* and P*rpsL*, the regulatory region of the y4wAB operon is (like that of the two *nifHDK* operons) strongly expressed inside bacteroids of NGR234. The transcription start site of y4wA was determined by 5′ RACE-PCR and found to be 10 bp downstream of the predicted binding site for σ^{54}. When cloned in the promoterless pRG960 reporter system, the 169 bp *Pst*I–*Bam*HI fragment that includes the NifA UAS, the consensus sequence for binding of RpoN and the predicted ATG of y4wA was shown to be sufficient to direct the expression of Gus inside nodules. Because of its specific endosymbiotic activity and its short length, which reduces the probability of undesired recombination in NGR234, PwA was selected as the reference promoter to be included in the new transposon Tn*EKm-PwA*. Previous attempts to generate non-polar mutants in symbiotic operons of, for example *Bradyrhizobium japonicum* (Fischer *et al.*, 1986), often relied on the transcriptional read-through from the strong constitutive promoter of the antibiotic-resistance gene carried by the mutagenizing cassette. As the native NifA-σ^{54}-dependent promoter remains silent until bacteria reach the nodule environment, this feature of Tn*EKm-PwA* dramatically reduces the risks of pleiotropic effects resulting from the deregulation of the loci targeted by mutagenesis. The analysis by qRT-PCR showed that in NGR117 the expression levels of genes upstream and downstream of Tn*EKm-PwA* were close to wild-type levels, whereas y4vGHIJ was silent in mutant NGR118, in which Tn*EKm-PwA* was inserted in reverse orientation (Table 4). 5′ RACE-PCR also confirmed that PwA activity was not affected by the terminal inverted repeats of the transposon since the transcription start of y4vG in strain NGR117 was found 10 bp downstream of the consensus σ^{54}-binding site embedded in Tn*EKm-PwA*. To test whether PwA retained its NifA-σ^{54}-dependent promoter activity in genetic backgrounds other than NGR234, construct pYXPwA was mobilized into *S. fredii* strain USDA257 by triparental mating. The strong Gus activity detected in *V. unguiculata* nodules formed by the resulting USDA257 transconjugant strain (data not shown) indicated that PwA retained its bacteroid-specific activity in other sinorhizobia. This result opens up the possibility to use Tn*EKm-PwA* to target NifA-regulated loci in genetic backgrounds other than NGR234.

In addition to the Fix$^-$ and pleiotropic phenotype of the *nifA*-deletion mutants of NGR234, our results obtained with strains mutated with either the Ω interposon cassette or the Tn*EKm-PwA* transposon clearly showed that none of the genes of the *nifQdctA1*y4vGHIJ operon is essential for symbiotic nitrogen fixation. This operon includes ORFs predicted to code for a member of the cytochrome P450 family (y4vG), one uncharacterized protein (y4vH), a short-chain dehydrogenase/reductase (y4vI) and a putative monooxygenase oxidoreductase (y4vJ), as well as *dctA1* and *nifQ*. *Rhizobium* sp. strain BR816 was reported to encode homologues of y4vGHIJ, the former of which (the product of ORF412 or CYP127A2) was shown to be strongly expressed inside bacteroids found in nodules of *Phaseolus vulgaris* and *L. leucocephala* (Luyten *et al.*, 2001). The symbiotic properties of the *cyp127A2* mutant strain of BR816 (strain FAJ1403) were not altered on its natural host *Leucaena* spp., while nodulation tests on *Phaseolus* cultivars showed a diminished acetylene reduction activity on *P. vulgaris* 'Negro Jamapa'. However, no significant differences were found in the nodule number, nodule fresh weight or shoot dry weight of the various host plants inoculated with either NGRΩy4vG or its parent strain. As sequence analysis confirmed that pSfrNGR234a carries additional cytochrome P450 loci in the form of *cpxP* (y4lD), *cpxR* (y4lC) and *cpxU* (y4kV) (Freiberg *et al.*, 1997), gene redundancy might be the reason for the absence of a statistically significant difference in the symbiotic performances of the mutant strains. *cpxDRU* belong to a single NifA- and σ^{54}-regulated operon that is strikingly similar at the nucleotide level to a locus of *B. japonicum* which is expressed symbiotically (Tully & Keister, 1993) and was proposed to be involved in the synthesis of gibberellins (Tully *et al.*, 1998). Even though these genes were shown to be conserved in many strains of the *Rhizobiaceae* (Keister *et al.*, 1999), they are not essential for the symbiosis of *B. japonicum* on soybean (Tully & Keister, 1993). Taken together, these results indicate that cytochrome P450 genes that are expressed within bacteroids are not necessarily essential to nitrogen fixation, and may represent adaptations to specific nodule niches rather than conserved components of the fixation apparatus. The finding that the *dctA1* mutant NGRΩ*dctA1* induced Fix$^+$ nodules was unexpected. Genome analysis predicted that, in addition to the conserved *dctABD* locus of pSfrNGR234b, the chromosome and megaplasmid of NGR234 code for a number of putative dicarboxylate transporters that may complement the *dctA1* mutation (Schmeisser *et al.*, 2009). Clearly, additional experiments are required to define the transporter(s) of NGR234 responsible for the uptake of C_4-dicarboxylate in bacteroids. The discrepancy with the reported symbiotic-deficient phenotype of NGR*dc1* can not be attributed to host specificity, as both NGR*dc1* and NGRΩ*dctA1* were tested on the same plant species (*V. unguiculata*, *M. atropurpureum* and *L. leucocephala*). According to van Slooten *et al.* (1992), NGR*dc1* was a deletion mutant in which a 475 bp *Cla*I–*Sst*I fragment located upstream of *dctA1* was replaced with the Ω interposon. A detailed analysis of the pSfrNGR234a sequence confirmed that the deleted fragment of NGR*dc1* replaced with Ω corresponded to most of the *nifQ* sequence instead of the *dctA1* promoter as was predicted by van Slooten *et al.* (1992). Conserved in most rhizobia, NifQ specifically donates molybdenum for the synthesis of the FeMo cofactor of the nitrogenase (Hernandez *et al.*, 2008). The Fix$^+$ phenotype of NGRΔ*nifQ* on *L. leucocephala* and

V. unguiculata suggests that inside these nodules of either determinate or indeterminate type, the concentration of molybdenum accessible to bacteroids is sufficient to sustain an active synthesis of nitrogenase and the establishment of a proficient symbiosis. Absolute requirement for *nifQ* in symbiotic nitrogen fixation was not necessarily expected as both *S. meliloti* 1021 and *R. leguminosarum* 3841 apparently lack a functional copy of *nifQ* (Masson-Boivin *et al.*, 2009). That the absence of NifQ in NGR234 had no effect on *V. unguiculata* development, and only reduced by 30 % the symbiotic performance of the *nifQ* mutant on *L. leucocephala*, further substantiates the need to determine the respective importance in distinct symbiotic contexts (i.e. host plants) of NifA-regulated loci. In this respect, the non-polar Tn*EKm-PwA* mutagenesis system will help us assess the contribution of each of the bacteroid-specific loci to the endosymbiotic lifestyles of the promiscuous strain NGR234.

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