Stable fluorescent and enzymatic tagging of *Bradyrhizobium diazoefficiens* to analyze host-plant infection and colonization

Raphael Ledermann, Ilka Bartsch, Mitja N. Remus-Emsermann*, Julia A. Vorholt, and Hans-Martin Fischer¹)

ETH Zurich, Institute of Microbiology, Vladimir-Prelog-Weg 4, CH-8093 Zurich, Switzerland

*Current address: Federal Department of Economic Affairs, Education and Research EAER, Agroscope, Institute for Food Sciences IFS, Schloss 1, P.B., 8820 Wädenswil, Switzerland

¹) Corresponding author:

Telephone +41 44 632 44 19; Fax: +41 44 632 13 78; E-Mail:fischeha@ethz.ch
(ABSTRACT)

*Bradyrhizobium diazoefficiens* USDA 100 (formerly named *Bradyrhizobium japonicum*) can fix dinitrogen when living as endosymbiont in root nodules of soybean and some other legumes. Formation of a functional symbiosis relies on a defined developmental program mediated by controlled gene expression in both symbiotic partners. In contrast to other well studied *Rhizobium*-legume model systems which have been thoroughly examined by means of genetically tagged strains, analysis of *B. diazoefficiens* host infection has been impaired due to the lack of suitable tagging systems. Here we describe the construction of *B. diazoefficiens* strains constitutively expressing single-copy genes for fluorescent proteins (eBFP2, mTurquoise2, GFP+, sYFP2, mCherry, HcRed) and enzymes (GusA, LacZ). For stable inheritance, the constructs were recombined into the chromosome. Effectiveness and versatility of the tagged strains was demonstrated in plant infection assays: (i) The infection process was followed from root hair attachment to colonization of nodule cells with epifluorescent microscopy. (ii) Monitoring mixed infections with two strains producing different fluorescent proteins allowed rapid analysis of nodule occupancy and revealed that the majority of nodules contained clonal populations. (iii) Microscopic analysis of nodules induced by fluorescent strains provided evidence for host-dependent control of *B. diazoefficiens* bacteroid morphology in nodules of *Aeschynomene afraspera* and *Arachis hypogaea* (peanut) as deduced from their altered morphology compared with bacteroids in soybean nodules.
(INTRODUCTION)

Formation of the intimate symbiosis between rhizobia and their legume host plants follows a complex, strictly controlled developmental program which eventually leads to the massive colonization of specialized plant organs, root nodules, by nitrogen-fixing endosymbionts (for reviews, see (Oldroyd et al., 2011; Downie, 2014). A number of model systems have been studied intensively in the past at the cellular, biochemical, and molecular genetic levels of both symbiotic partners. The analysis of infection and colonization processes was greatly stimulated by the use of light microscopy in combination with genetically tagged rhizobial strains that express fluorescent or enzymatic proteins (Gage, 2002; Oldroyd and Downie, 2008). While such tagged rhizobial strains are well established for model systems like *Sinorhizobium* spp. – *Medicago* spp. or *Mesorhizobium* spp. – *Lotus* spp. and others (*Stuurman et al., 2000*) similar tools are largely lacking for the soybean symbiont *Bradyrhizobium diazoefficiens* USDA 100 (formerly named *Bradyrhizobium japonicum*) (*Delamuta et al., 2013*) despite the agricultural significance of and scientific interest in the latter system. In previous investigations with the *B. diazoefficiens*-soybean symbiosis staining techniques and electron microscopy were used to visualize the endosymbionts at different stages of the infection process (*Pueppke, 1983; Turgeon and Bauer, 1985*). Lack of endogenous plasmids in *B. diazoefficiens* and the rapid loss even of broad-host range plasmids in the absence of selective pressure (*Alvarez-Morales et al., 1986; Stuurman et al., 2000*) have hampered the development of versatile plasmid-based tagging tools that are widely used in other systems. Chromosomal integration of respective marker genes offers an alternative for stable genetic tagging. In fact, transposons have been used to integrate genes for fluorescent proteins in different Alphaproteobacteria including strains of *Bradyrhizobium* (*Matthysse et al., 1996; Bhatia et al., 2002; Okubo et al., 2013; Hayashi et al., 2014; Schada...*)
von Borzyskowski et al., 2015). However, this strategy yielded random chromosomal
insertions which may have undesired secondary effects depending on the location of the
inserted transposon. To circumvent this limitation, Tn7-based marker-delivery systems were
developed which enabled site-specific insertion of markers at a single attTn7 site present in
different Gram-negative bacteria (Koch et al., 2001; Choi et al., 2005). However, since this
system turned out to work inefficiently in *B. diazoefficiens* (own unpublished results) we
have designed an integrative vector serving as a basis for stable tagging of *B. diazoefficiens*
with either one of six fluorescent or two enzymatic proteins. The respective non-replicative
plasmids were introduced by conjugation into *B. diazoefficiens* and chromosomally
integrated via homologous recombination in a symbiotically dispensable region. Appropriate
transcription of the single-copy marker genes was ensured by the use of strong constitutive
promoters. Here, we describe the construction of this novel genetic toolset for *B.
*diazoefficiens* and demonstrate its utility for the cellular analysis of developmental steps
during formation of the symbiotic interaction between *B. diazoefficiens* and host plants. In
another application we used differently tagged strains for mixed infection assays and
assessed nodule occupancy based on the distinct fluorescent properties of the inoculated
strains.

RESULTS AND DISCUSSION

Chromosomal integration of single-copy genes encoding fluorescent or enzymatic proteins
in *B. diazoefficiens*.

We constructed 13 derivatives of *B. diazoefficiens* strain 110spc4, which constitutively
express fluorescent (11) or enzymatic (2) protein tags from single copy genes integrated in
the chromosome (Table 1). Chromosomal integration was crucial because of the notorious
tendency of *B. diazoefficiens* to lose plasmids in the absence of antibiotic selection, e.g., in root nodules. All constructs were chromosomally integrated at the same symbiotically dispensable region downstream of the *scoI* gene (Bühler et al., 2010). Comparative soybean infection tests with tagged strains and untagged wild type revealed that integration of plasmids at this locus neither affected symbiotic properties nor nodulation competitiveness (Fig. S1). Two different, constitutive promoters (Paph originating from the kanamycin resistance gene *aphII* of Tn5 and PtuF of the gene for elongation factor Tu of *Methylobacterium extorquens* AM1) were tested for driving transcription of four of the seven genes encoding fluorescent proteins. Because all genes were present in single copy only, the rather strong Paph and PtuF promoters were chosen to ensure appropriate fluorescence intensities. The latter promoter was characterized previously and shown to function efficiently in *B. diazoefficiens* (Schada von Borzyskowski et al., 2015). While our previously generated transcriptomics data suggested that the promoter of the *B. diazoefficiens* *rrn* operon is probably even stronger (Hauser et al., 2007; Pessi et al., 2007) we refrained from using it here because very high levels of tagging proteins may result in undesired secondary effects.

Comparison of fluorescence intensity derived from mCherry expressed from Paph or PtuF revealed no significant differences between the two promoters (data not shown). Different fluorophores, however, exhibited different fluorescence intensities in *B. diazoefficiens* (Fig. 1A). Because fluorescence of *B. diazoefficiens* strain GFP-1 was rather weak, the codon composition of the respective GFP+ gene was optimized according to the codon frequency of *B. diazoefficiens* (Ramseier and Göttfert, 1991; Grote et al., 2005). The resulting synthetic *bjGFP* gene (see Fig. S2 for nucleotide sequence) was placed under the control of Paph and
integrated into the chromosome to yield strain bjGFP-1. Cells of this strain exhibited a drastically increased fluorescence compared to strain GFP-1 (data not shown).

Based on fluorescence intensity, photo-bleaching susceptibility and plant tissue autofluorescence, we found strains expressing mCherry (mChe-1, mChe-4), sYFP2 (sYFP2-1, sYFP-4) or bjGFP (bjGFP-1) the most useful. However, for visualization of *B. diazoefficiens* cells in plant tissue which contains plastids exhibiting red autofluorescence (*e.g.*, stem nodules of *Aeschynomene afraspera*), the use of blue (eBFP2-1, eBFP2-4) or cyan (mTq2-1, mTq2-4) tagged bacteria may be superior with eBFP2 being less favorable because of its pronounced susceptibility to photobleaching.

To characterize the excitation and emission spectra of the constructed *B. diazoefficiens* strains, spectra of respective cell suspensions were recorded (Fig. 1B). The excitation and emission maxima were as follows (in nm): 385/450 (strain eBFP2-1), 456/474 (mTq2-1), 497/509 (bjGFP-1), 505/540 (sYFP2-1), 590/606 (mChe-1), and 591/635 (HcRed-1). When signal intensities of the emission maxima were normalized to the number of cells present in the suspensions the following order in fluorescence intensity was found: sYFP2-1 > mTq2-1 > eBFP2-1 > bjGFP-1 > mChe-1 > HcRed-1 (Fig. 1A).

Notably, the excitation spectrum of mTq2-1 cells exhibited two local maxima (435 and 456 nm) as described also for the purified protein (Goedhart et al., 2012). Unlike the purified protein, however, excitation absorption of mTq2-1-tagged cells was more pronounced at 456 nm than at 435 nm in *B. diazoefficiens*. Similarly, HcRed-1 cells had two emission peaks with the more pronounced one at 635 nm blue-shifted by 5 nm as compared with the purified HcRed protein (Gurskaya et al., 2001) and a minor one around 618 nm, which was specified as maximum for HcRed in another study (Day and Davidson, 2009). Minor differences between the *in vitro* and *in vivo* spectral properties of fluorescent proteins are
not uncommon and likely due to different physical-chemical conditions prevailing in solution and in cells (Mylle et al., 2013). For example, the presence of pigments or other chromophores in particular cells may affect the spectral features of fluorescent proteins.

Visualization of early infection stages.

Tagged *B. diazoefficiens* strains were used to visualize root-hair attachment and early stages of soybean infection. Strains mChe-1 (or mChe-4 in some experiments), bjGFP-1 and GusA-1 were inoculated on soybean seedlings which were then incubated for 4 days before epidermal cell layers of roots were peeled off and inspected by epifluorescence microscopy or stained with X-gluc (5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid). Individual fluorescent bacteria were readily detectable, mostly being attached in a polar manner (Fig. 2A). Colonization of roots was not uniform but most pronounced in zones with dense root hairs, whereas only a few bacteria were attached on epidermal cells (not shown).

Soybean seedlings are infected by *B. diazoefficiens* via thin infection threads emerging from micro-colonies entrapped between short deformed root hairs and epidermal cells (Pueppke, 1983; Turgeon and Bauer, 1985). With the help of tagged strains, bacteria-containing infection threads could be observed in great detail. Soon after penetration of the root hair, infection threads began to branch leading to several threads within a single root hair cell (Fig. 2B). Given the thin nature of soybean infection threads invading bacteria were lined up in a file of individual cells (Fig. 2C).

Infection threads in adjacent root hair cells were mostly populated with only one type of bacterium when GFP- and mCherry-tagged strains were inoculated simultaneously (Fig. 2D). However, in rare cases when entrapped micro-colonies consisted of mixed populations we also observed root hairs with multiple infection threads harboring either strain (Fig. 2E).
Notably, we never detected individual infection threads harboring more than one strain while analyzing approximately 50 infection threads on each of two soybean plants. This is different from what has been described for *Medicago sativa* and vetch where different bacterial strains of *S. meliloti* and *Rhizobium leguminosarum* bv. *viciae*, respectively, co-existed in a single infection thread, which may be related to the thicker nature of infection threads in those hosts (Gage et al., 1996; Stuurman et al., 2000; Gage, 2002). While infection threads of *M. sativa* ramify only after reaching the cortical cell layers, soybean root hair cells frequently showed multiple infection threads which may have originated from early branching or multiple, independently formed threads. The latter situation may explain the formation of mixed infected nodules described below.

An application of GusA-tagged bacteria is illustrated in Fig. 3F showing an overview of a longitudinal section that was cut from a soybean root 4 days after inoculation with *B. diazoefficiens* strain GusA-1. Localization of emerging nodule primordia which are characterized by small, anticlinal dividing cells is facilitated by the dark precipitated product resulting from X-gluc hydrolysis by the GusA-tagged bacteria present in infection threads.

**Use of tagged strains to study nodule occupancy.**

We have used *B. diazoefficiens* strains mChe-4 and sYFP2-4 in mixed-infection experiments to study colonization of soybean, cowpea, mung bean, and siratro nodules. These strains, which express the respective fluorescent proteins from the PtuF promoter, were readily detected in infected host cells (Fig. 3). This documents that the PtuF promoter is equally well suited as the Paph promoter to drive transcription of the genes encoding fluorescent tags. Based on epifluorescence microscopy, four different colonization patterns could be distinguished. The vast majority of all inspected nodules were colonized by only one of the
two strains supporting the idea that rhizobial populations in legume nodules are largely
clonal (Simms et al., 2006; Pobigaylo et al., 2008) (Fig. 3A; see also Fig. 4). In some of the
analyzed nodules, however, both strains were present. The two populations were either
separated by uninfected cells suggesting that these nodules originated from two
independent but spatially close infection events ("fused nodules"; Fig. 3B) or present in
immediately adjacent host cells ("mixed nodules"; Fig. 3C). Mixed nodules might be the
result of simultaneous entry of both strains present in branched infection threads in
individual root hair cells (cf. Fig. 2E). At a very low frequency individual host cells harboring
both symbionts were detected in a few nodules (arrowheads in Fig. 3D). Based on the results
described above we assume that the different symbionts entered these host cells via distinct
infection threads.

Enzymatically tagged strains GusA-1 and LacZYA-1 turned out to be very useful for obtaining
a rapid overview of nodule occupancy (Fig. 3E and F). In a mixed infection experiment with
these strains, nodules populated by one or both strains were readily distinguished by
simultaneous staining with X-gluc and Green-β-D-gal (1-Methyl-3-indolyl-β-D-
galactopyranoside).

We also performed quantitative mixed infection tests with different hosts including three
soybean varieties (green butterbean, Williams 82, black jet) and three additional legume
species Vigna radiata (mungbean), Vigna unguiculata (cowpea) and Macroptilium
atropurpureum (siratro). For inoculation, 1:1 or 10:1 mixtures of B. diazoefficiens strains
expressing mCherry or sYFP2 containing a constant total number of $10^3$ cells (Fig. 4A, B) or
1:1 mixtures containing $10^2$, $10^4$, $10^6$, $10^8$, or $10^{10}$ total cells (Fig. 4C) were used. As described
above for soybean cv. green butterbean, the great majority of the nodules were colonized by
only one strain regardless of the host plant, and only few fused or mixed nodules were
detected (Fig. 4A). The ratio of mChe-4- to sYFP2-4-only populated nodules reflected the ratio of the strains in the inoculum, i.e., about 1:1 and 10:1 in the experiments shown in Fig. 4A and 4B. Increasing cell numbers in 1:1 inoculum mixtures revealed no differences with regard to the macroscopic plant phenotype and total number of nodules, regardless of the inoculum size (data not shown). Moreover, the ratio between different nodules types was roughly stable when $10^2$, $10^4$, $10^6$, or $10^8$ cells were inoculated per plant (Fig. 4C). Only when as many as $10^{10}$ bacteria were applied per seedling, a slight increase of fused nodules was observed, yet even under these high-density inoculation conditions >50% of all nodules were populated with only one strain.

Fluorescently tagged rhizobia have been used previously to study formation and colonization of mixed infected nodules. Examples include the Sinorhizobium meliloti – Medicago sativa or Bradyrhizobium sp. ORS285 – Aschynomene spp. symbioses which differ in the mode of host entry (infection thread-dependent vs. crack entry) and the type of nodules (indeterminate vs. determinate) (Gage, 2002; Bonaldi et al., 2011). To our knowledge this is the first study where fluorescently tagged B. diazoefficiens strains were used to follow early infection stages and colonization of soybeans and related legumes which all are infected via threads to develop determinate nodules. A notable result of the mixed infection experiments was our observation that the majority of nodules contain clonal populations even when a very dense inoculum was applied. This finding combined with the high spatial density of primary infection sites and emerging nodule primordia (Fig. 2F) suggests that a large proportion of primary infection events are abortive, which is in agreement with similar observations made previously by (Calvert et al., 1984).

B. diazoefficiens bacteroid morphology is host-dependent.
For indeterminate symbioses it is well established that bacteroid differentiation, including morphological alterations, is dependent on the host plant (Mergaert et al., 2006; Oono et al., 2010; Kondorosi et al., 2013). It was previously shown for the photosynthetic *Bradyrhizobium* sp. ORS285 that bacteroids of determinate *Aeschynomene* legume nodules may either be rod shaped (though enlarged) in *A. afraspera* but spherical in *A. indica* (Bonaldi et al. 2011). The most prominent host of *B. diazoefficiens* is soybean which belongs to the Millettioid clade of legumes and forms determinate nodules containing rod-shaped bacteroids that are morphologically very similar to free-living cells. In addition to soybean, *B. diazoefficiens* is able to induce determinate nodules on several other host plants including *V. radiata*, *V. unguiculata*, *M. artropurpureum*, *A. afraspera*, and *Arachis hypogaea* (peanut) (Göttfert et al., 1990a; Renier et al., 2011; Noisangiam et al., 2012). Yet, little is known about the morphology of *B. diazoefficiens* bacteroids in the latter two species which belong to the Dalbergioid clade of legumes. We used mCherry-tagged *B. diazoefficiens* in combination with confocal fluorescence microscopy to compare the morphology of bacteroids present in nodules of soybean, *A. afraspera* and peanut (Fig. 5). While bacteroids in soybean nodules were small and rod-shaped like free-living cells (Fig. 5A), we found that bacteroids in nodules of peanut and *A. afraspera* underwent morphological changes. While bacteroids in *A. afraspera* nodules retained a rod-like morphology most of them were enlarged to varying extents (Fig. 5B). Most strikingly, the development of bacteroids in peanut nodules was accompanied by massive swelling resulting in large spherical bacterial cells (Fig. 5C) like it has been described for “cowpea rhizobia” when present in peanut nodules (Chandler, 1978; Sen et al., 1986). Thus, the concept of host-controlled bacteroid morphology also applies to *B. diazoefficiens*. It remains to be determined whether or not the increased bacteroid size in *A. afraspera* and peanut nodules is accompanied by additional alterations such as decreased
cell viability or elevated DNA content that are both characteristic for bacteroids in indeterminate nodules.

Conclusions.

The plasmids and strains described in this work represent long desired additions to the molecular toolbox of *B. diazoefficiens*, the symbiont of the agriculturally important host soybean. For reasons described in the introduction, all marker genes were stably integrated in the chromosome at a uniform locus without discernable phenotypic consequences other than the tag and the tetracycline resistance associated with it. Although expression was driven from a marker gene in single copy, strong transcription and translation was achieved in *B. diazoefficiens*. Cells tagged with fluorescent or enzymatic marker genes under the control of the Paph or Ptuf promoter were readily detected at all stages of symbiosis with different hosts documenting the effectiveness of the constructed strains. The constructs made in this study allowed for the first time to visualize autofluorescent *B. diazoefficiens* cells in soybean infection threads, which is particularly challenging given the thin, short and early branching nature of soybean infection threads compared to other rhizobial systems. Moreover, the genetically tagged strains enabled rapid analysis of nodule occupancy and provided evidence for host-dependent control of *B. diazoefficiens* bacteroid morphology. These promising results suggest that the tools developed in this study may become particularly useful for the characterization of symbiotically impaired mutants at the level of host infection, colonization, and competitiveness.

Beyond tagging of *B. diazoefficiens* as described here, the plasmids developed herein may also prove useful for other bacteria after replacing the respective *B. diazoefficiens* DNA region with a suitable DNA fragment from the organism of interest. To do so, the dedicated
flanking restriction sites can be used to excise the *B. diazoefficiens* DNA and introduce an appropriate piece of DNA (see Fig. S3). In fact, this strategy has been successfully used for chromosomal insertion of pRJPtuf-mCherry derivatives in three other alphaproteobacterial species, *i.e.*, *Rhodopseudomonas palustris* TIE-1, *M. extorquens* AM1, and *Sphingomonas melonis* FR1 (unpublished) indicating that the genetic tools described offer applications beyond the use in *B. diazoefficiens*. 
MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown in LB medium (Miller, 1972) at 37 °C. Antibiotics were used at the following concentrations (µg/ml): ampicillin (200), gentamicin (10), tetracycline (10). *B. diazoefficiens* strains were grown at 30 °C in PSY medium supplemented with 0.1% L(+)-arabinose (Mesa et al., 2008) and appropriate antibiotics (µg/ml): spectinomycin (100), tetracycline (50 in agar, 25 in liquid cultures), chloramphenicol (20; for counterselection of *E. coli*). Fluorescent cells used for recording excitation and emission spectra were grown in MOPS-buffered minimal medium (Guerinot et al., 1990). Additional details related to strains and plasmids are provided in Table S1.

Construction of tagged *B. diazoefficiens* strains.

All *B. diazoefficiens* strains harboring genetically encoded fluorescent tags were constructed according to a uniform strategy, which is described in detail only for strain GFP-1. For further details about construction of analogous strains, see Supplemental Material. Plasmid pRJ2575 was digested with *Eco*RI and *Pst*I releasing a linear DNA fragment which consists of the conjugative vector pSUP202pol4 (Fischer et al., 1993) plus 807 bp *B. diazoefficiens* DNA originating from the region downstream of the *scol* (blr1131) gene (Bühler et al., 2010). This construct was ligated with a 1104 bp *Eco*RI-*Pst*I fragment isolated from plasmid pMG103-npt2-GFP-BisAH-DG1 (Bonaldi et al., 2010) which contains a transcriptional fusion of the promoter of the *aphII* kanamycin resistance gene (Paph) to the GFP+ gene (Scholz et al., 2000; Karunakaran et al., 2005). The resulting plasmid pRJPaph-gfp was transformed into *E. coli* strain S17-1 λpir from where it was transferred to *B. diazoefficiens* by biparental...
conjugation. Upon selection for tetracycline resistance, clones were obtained which harbored the entire plasmid integrated in the *B. diazoefficiens* chromosome by homologous recombination downstream of *scoI*. Correct integration at the locus was verified by PCR amplification of DNA regions spanning the recombination site using primers listed in Table S2.

For generation of all other fluorescently tagged *B. diazoefficiens* strains listed in Table 1, the respective plasmids were constructed analogously to pRJPaph-gfp (for details, see Supplemental Material and Fig. S3) and integrated into the *B. diazoefficiens* chromosome downstream of *scoI*. In addition to Paph, we also used the promoter associated with the thermo-unstable (Tu) translation elongation factor gene of *Methyllobacterium extorquens* AM1 (PtuF) to drive transcription of selected fluorescent protein-encoding genes (Schada von Borzyskowski et al., 2015). The respective constructs PtuF-eBFP2, PtuF-mTurquoise2, PtuF-YFP2, and PtuF-mCherry (Supplemental Material) were chromosomally integrated downstream of *scoI* as described above for the Paph-driven constructs.

Finally, we also constructed two enzymatically tagged *B. diazoefficiens* strains (lacZYA-1 and gusA-1) containing constitutively expressed lacZYA and gusA genes. They were obtained by integration via homologous recombination of plasmids pRJPaph-lacZYA and pRJPaph-gusA (Supplemental Material), respectively, into the chromosome of *B. diazoefficiens* wild type downstream of *scoI*.

**Excitation and emission spectra of tagged *B. diazoefficiens* cells.**

Fluorescent spectra of cell suspensions were recorded with a Cary Eclipse spectrofluorimeter (Agilent Technologies, Santa Clara, CA, USA). All measurements were done using the following settings: 5 nm excitation and emission slits, 30 nm/min scan rate, and 1000 V PMT.
voltage. Fluorescently tagged bacteria were cultivated in minimal medium harvested by centrifugation at early stationary phase, washed twice in PBS and adjusted to OD$_{600}$ = 0.75, except for strain sYFP2-1 which was adjusted to OD$_{600}$ = 0.15 because of its high fluorescence. While recording spectra, wavelengths of excitation and emission were offset from the optimal values in order to minimize interference with scattered excitation light. Specifically, spectra were recorded at the following wavelengths (excitation/emission in nm):

- 385/450 (strain eBFP2-1)
- 435/490 (mTq2-1)
- 480/520 (bjGFP-1)
- 505/540 (sYFP2-1)
- 540/625 (mChe-1)
- 550/635 (HcRed-1)

Spectra represent means of 3 recordings (HcRed-1 emission: 5). Spectra were normalized to the respective maximal excitation and emission. To quantify the maximal fluorescent intensity, all strains were excited at their respective maxima, and point measurements at optimal emission wavelengths were recorded. Fluorescent intensity then was normalized to cfu in the measured culture.

**Plant inoculation tests.**

Seeds of soybean (*Glycine max* (L.) Merr.) cv. Williams 82 (kindly provided by D.-N. Rodriguez, CIFA, Las Torres-Tomejil, Seville, Spain), cv. black jet and cv. green butterbean, mung bean (*Vigna radiata* (L.) R. Wilczek), cowpea (*Vigna unguiculata* (L.) Walp.) cv. Iron & Clay (Johnny's Selected Seeds, Albion, ME, USA), siratro (*Macroptilium atropurpureum* (DC.) Urb.) (kindly provided by W. D. Broughton, University of Geneva, Switzerland), and peanut (*Arachis hypogaea* L.; TRS Wholesale Co. Ltd. Southall, Middlesex, UK) were surface sterilized by immersing in 100% ethanol for 5 min and 35% H$_2$O$_2$ for 15 min. After intensive washing with sterile water, plant tests were performed as described previously (Göttfert et al., 1990b). Seeds of *A. afraspera* J. Léonard (kindly provided by E. Giraud, Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), Montpellier, France) were surface
sterilized in concentrated sulfuric acid for 45 min, washed, soaked for 24 h in sterile water
and processed subsequently as described above, except that plants were grown under
waterlogged conditions. To obtain stem nodules, a previously described procedure was
applied (Renier et al., 2011).

Sample preparation and fluorescent microscopy.

Nodules were routinely harvested 21 days post inoculation (dpi), except for siratro (28 dpi)
and peanuts (35 dpi), fixed in 4% formalin in phosphate-buffered saline (1.9 mM NaH₂PO₄,
8.1 mM Na₂HPO₄, 140 mM NaCl) at 4 °C overnight, washed twice in PBS supplemented with
50 mM NH₄Cl, dehydrated in 20% (w/v) sucrose in PBS at 4 °C for 24 h, briefly rinsed in 80%
ethanol and embedded in TissueTek® O.C.T.™ Compound (Sakura Finetek Europe B.V.,
Alphen aan den Rijn, The Netherlands) before shock freezing in liquid nitrogen. Semi-thin
sections of 10 µm were prepared by using a Microtome Cryostat HM525 (MICROM
International GmbH, Walldorf, Germany) and mounted on glass slides. Slides were washed in
PBS, air dried and mounted in 50% glycerol before microscopic examination. To acquire high-
quality micrographs, sections were mounted with the antifade mounting resin citifluor AF1
(Citifluor Ltd., London, UK). Epifluorescence microscopy was performed using Zeiss filter
sets: 13 (GFP/sYFP2), 15 or 43 (mCherry). At least 3 non-consecutive sections per nodule
were examined to evaluate the bacterial colonization.

For observation of root hair attachment and infection threads, roots were harvested 4 dpi,
rinsed in water to remove vermiculite particles and washed as described elsewhere
(Bulgarelli et al., 2012). Epidermal cell layers of roots were peeled off using a blunt razor
blade and mounted on glass slides in 50% glycerol.
High-resolution images of bacteroids were recorded using confocal microscopy. For soybean and peanut bacteroids, a Leica SP2-FCS point laser scanning confocal microscope (Leica microsystems AG, Heerbrugg, CH) was used. mCherry was excited with a HeNe laser at 594 nm. Fluorescence emission was recorded between 600 and 648 nm. For enhanced contrast, plant tissue autofluorescence was excited at 514 nm with an argon laser and emission was recorded between 520 and 560 nm. Bacteroids in A. afraspera nodules were imaged using a Visitron spinning disc confocal system (Visitron Systems GmbH, Puchheim, Germany) on a Zeiss 200m microscope. Bacteroids, expressing mCherry were excited at 561 nm and emission recorded with an ET 605/52m filter (Chroma Technology, Bellows Falls, VT, USA).

**Staining for GusA and LacZ activity.**

For observations of early infection stages, roots of *B. diazoefficiens* GusA-1-inoculated soybean plants were harvested 4 dpi and washed as described above. GusA activity in bacteria present in infection threads was visualized according to a modified staining protocol described previously (Wilson et al., 1995). In short, roots were placed in staining solution (19.5 mM NaH$_2$PO$_4$, 30.5 mM Na$_2$HPO$_4$, 1mM EDTA, 1mM K$_3$[Fe(CN)$_6$], 0.1% Triton X-100, 0.1% sarkosyl) and 200 µg/ml X-gluc, vacuum-treated for 20 min and incubated 12 h at 37°C. Subsequently, roots were cleared with chloral hydrate (Hayashi et al., 2005), sliced longitudinally and observed by differential interference contrast (DIC) light microscopy. For simultaneous detection of GusA and LacZ activity in mixed infected soybean nodules, these were harvested 23 dpi, detached from roots, washed in PBS amended with 0.02% Silvet L-77, cut in half, washed again briefly in PBS, and placed in the staining solution.
described above. X-gluc and Green-β-D-gal (both purchased from Biosynth, Staad, Switzerland) were added to a concentration of 100 µg/ml each, and nodules were vacuum-treated for 30 min followed by incubation at 28 °C for 4 h before they were observed under a stereo microscope. Endogenous plant-derived β-galactosidase activity was detected only after prolonged incubation, i.e., it did not interfere with LacZ-derived activity, and there was no need for its inhibition by preceding glutaraldehyde fixation.

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Author contributions

RL, MNR, JAV and HMF conceived the study and designed experiments. RL, IB, MNR and HMF executed experiments and analyzed data. RL, MNR and HMF wrote the manuscript.

LITERATURE CITED


Göttfert, M., Hitz, S., and Hennecke, H. 1990b. Identification of *nodS* and *nodU*, two inducible genes inserted between the *Bradyrhizobium japonicum* *nodYABC* and *nodIJ* genes. Mol. Plant Microbe Interact. 3:308-316.


the soil oligotrophic bacterium *Agromonas oligotrophica* (*Bradyrhizobium oligotrophicum*) is a nitrogen-fixing symbiont of *Aeschynomene indica*. Appl. Environ. Microbiol. 79:2542-2551.


of synthetic operons in *Methylobacterium extorquens* AM1 and other alphaproteobacteria.


**Table 1.** Bacterial strains and plasmids

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Relevant phenotype or genotype</th>
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<td><strong>Escherichia coli</strong></td>
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<td>supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 gyrA96 thi-1 relA2</td>
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<td>S17-1 λpir</td>
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<td>de Lorenzo et al., 1993</td>
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<td>Sp’ wild type (USDA 110 derivative)</td>
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sYFP2-1 Sp′ Tc′ 110spc4 with chromosomal integration of pRJPaph-sYFP2

mChe-1 Sp′ Tc′ 110spc4 with chromosomal integration of pRJPaph-mChe

HcRed-1 Sp′ Tc′ 110spc4 with chromosomal integration of pRJPaph-HcRed

eBFP2-4 Sp′ Tc′ 110spc4 with chromosomal integration of pRJPtuF-eBFP2

mTq2-4 Sp′ Tc′ 110spc4 with chromosomal integration of pRJPtuF-mTq2

sYFP2-4 Sp′ Tc′ 110spc4 with chromosomal integration of pRJPtuF-sYFP2

mChe-4 Sp′ Tc′ 110spc4 with chromosomal integration of
pRJPtuF-mChe

**GusA-1**  
Sp' Tc' 110spc4 with chromosomal integration of pRJPaph-gusA

**LacZYA-1**  
Sp' Tc' 110spc4 with chromosomal integration of pRJPaph-lacZYA

**Plasmids**

**pRJPaph-gfp**  
Tc' (pRJ2575) Paph-gfp+ for integration downstream of *scoil*

**pRJPaph-gfp_a1**  
Tc' (pRJPaph-gfp) replaced *PstI* site with *AatII* and *KpnI* sites

**pRJPaph-eBFP2**  
Tc' (pRJPaph-gfp_a1) Paph-eBFP2 for integration downstream of *scoil*

**pRJPaph-mTq2**  
Tc' (pRJPaph-gfp_a1) Paph- *mTurquoise2* for integration downstream of *scoil*

**pRJPaph-bjGFP**  
Tc' (pRJPaph-gfp_a1) Paph-*bjGFP* for integration
downstream of \textit{scol}

\textbf{pRJPaph-sYFP2} \quad \text{Tc}^{\prime} \ (\text{pRJPaph-gfp\_a1}) \ \text{Paph-sYFP2} \ for \ integration \quad \text{This work}

downstream of \textit{scol}

\textbf{pRJPaph-mChe} \quad \text{Tc}^{\prime} \ (\text{pRJPaph-gfp\_a1}) \ \text{Paph-mCherry} \ for \ integration \quad \text{This work}

downstream of \textit{scol}

\textbf{pRJPaph-HcRed} \quad \text{Tc}^{\prime} \ (\text{pRJPaph-gfp}) \ \text{Paph-HcRed} \ for \ integration \quad \text{This work}

downstream of \textit{scol}

\textbf{pRJPtuF-eBFP2} \quad \text{Tc}^{\prime} \ (\text{pRJPaph-gfp\_a1}) \ \text{Ptuf-eBFP2} \ for \ integration \quad \text{This work}

downstream of \textit{scol}

\textbf{pRJPtuF-mTq2} \quad \text{Tc}^{\prime} \ (\text{pRJPaph-gfp\_a1}) \ \text{Ptuf-mTurquoise2} \ for \ integration \quad \text{This work}

downstream of \textit{scol}

\textbf{pRJPtuF-sYFP2} \quad \text{Tc}^{\prime} \ (\text{pRJPaph-gfp\_a1}) \ \text{Ptuf-sYFP2} \ for \ integration \quad \text{This work}

downstream of \textit{scol}

\textbf{pRJPtuF-mChe} \quad \text{Tc}^{\prime} \ (\text{pRJPaph-gfp\_a1}) \ \text{Ptuf-mCherry} \ for \ integration \quad \text{This work}

downstream of \textit{scol}

\textbf{pRJPaph-gusA} \quad \text{Tc}^{\prime} \ (\text{pRJPaph-gfp\_a1}) \ \text{Paph-gusA} \quad \text{This work}
gusA for integration
downstream of scoI

pRJPaph-lacZYA

Tc' (pRJPaph-gfp) Paph-
lacZYA for integration
downstream of scoI

This work

576

577
FIGURE LEGENDS

Fig. 1. Fluorescence of free-living *B. diazoefficiens* cells harboring chromosomally integrated genes that encode different fluorescent proteins. **A**, Fluorescence of cell sediments irradiated with a 365 nm light source (above), and maximal fluorescence intensity of cell suspension (arbitrary units) deduced from the emission spectra shown in panel **B** and normalized to the cfu counts of the respective cell suspension (below). **B**, Excitation (dotted lines) and emission spectra (solid lines) of the indicated strains (OD$_{600}$ =0.75 for all strains except sYFP2-1 where the respective value was 0.15). Relative fluorescent intensities are shown with the maximum of each individual spectrum defined as 100%.

Fig. 2. Visualization of early stages during soybean infection by tagged *B. diazoefficiens* strains. Soybean seedlings were inoculated with *B. diazoefficiens* strains mChe-1 (red), bjGFP-1 (green) or GusA-1 and observed by fluorescence (**A-E**) or DIC microscopy (**F**) at 4 dpi. **A**, Polar attachment of mChe-1 cells (white arrow heads) to root hair cells. Thin infection threads containing mCherry- (**B**) or GFP-tagged bacteria (**C**) (exemplary bacterial cells marked with white arrow heads in both panels) tend to branch very early on during formation leading to a network. After co-inoculation of strains mChe-1 and bjGFP-1 (1:1 ratio; **D, E**) individual root hair cells were infected either by only one strain (**D**) or simultaneously by both strains via infections threads emerging from a mixed micro-colony (**E**). **F**, Visualization of emerging nodule primordia (small, anticlinal dividing plant cells) and associated infection threads by X-gluc staining of GusA-tagged bacteria (dark grey).

Fig. 3. Nodule colonization patterns observed after mixed infection of soybean seedlings with fluorescently or enzymatically tagged *B. diazoefficiens* strains. Micrographs **A-D** show
overlays of mCherry- and YFP-derived fluorescence from strains mChe-4 (red) and sYFP-4 (green). Nodules are colonized either by one strain (A; example sYFP2-4-colonized nodule) or by two (B, C, D). The latter type of nodules comprises “fused nodules” seemingly resulting from the fusion of two adjacent emerging nodules and retain uninfected cells (ui) between zones infected by either of both strains (B), and truly mixed infected nodules where no uninfected cells were present between zones populated by the two strains (C). In rare cases, individual host cells simultaneously harbored both strains (D; arrowheads). Panels E and F show sections through fused and clonal nodules, respectively, formed upon mixed infections with enzymatically tagged strains GusA-1 (blue) and LacZYA-1 (turquoise) and subsequent staining.

Fig. 4. Use of fluorescently tagged *B. diazoefficiens* strains to study nodule occupancy in dependence of host and inoculum composition. Strains mChe-4 and sYFP2-4 were mixed 1:1 (A) or 10:1 (B) at a constant total cell number of $10^3$ or 1:1 with the specified, increasing total cell numbers (C), and inoculated on the indicated legumes (A, B) or soybean cv. green butterbean (C). From each infection experiment, 10 spherical, well separated nodules of each of two plants were harvested and nodule occupancy was analyzed by fluorescent microscopy. Shown are percentages of nodules colonized by only one strain or both strains in “fused” or “mixed” nodules. For further details, see text and Fig. 3.

Fig. 5. Host-dependent morphology and size of *B. diazoefficiens* bacteroids. Confocal micrographs of mCherry-tagged *B. diazoefficiens* mChe-1 bacteroids present in infected cells of soybean (A), *Aeschynomene afraspera* (B), or peanut root nodules (C). Bacteroids are rod shaped in soybean, enlarged to various extent and hence irregularly shaped in *A. afraspera,*
or very large and spherical in peanut. To highlight the difference in size distinct bacteroids are labeled with white arrowheads in panels A and B.

e-Xtras.

**Supplemental Material.** Construction of tagged *B. diazoefficiens* strains

Table S1. Complete list of strains and plasmids used in this work.

Table S2. Oligonucleotides used in this study.

Fig. S1. Symbiotic properties of *B. diazoefficiens* strains 110spc4 (wild type), GFP-1 and GusA-1. Soybean cv. Williams 82 seedlings were inoculated with strain 110spc4 (n = 6) or GFP-1 (n = 5) and nodule number (A), nodule dry weight (B), and nitrogenase activity (C) were determined 21 dpi. (D) To assess competitiveness of strain GusA-1 against the wild type, both strains were mixed at the indicated ratios and 1 ml aliquots of the mixture (2*10^1–2*10^2 cells in total) were used to infect seedlings of soybean cv. green butterbean. For each inoculation ratio, nodules from two plants were detached from roots 20 dpi, cut in half, and nodule occupancy was analyzed by GusA staining as described in Materials and Methods. Numbers above columns indicate to the number of analyzed nodules. The fact that nodule occupancy largely reflected the ratio of both strains in the inoculum indicates that chromosomal insertion of tagging constructs does not affect competitiveness of tagged strains. Note that the data for the 50:50-mixed infection was generated in an independent experiment which was carried out separately from the others.
Fig. S2. Nucleotide sequence alignment of the original GFP+ gene (top line) and the codon-optimized bjGFP gene (second line) with exchanged nucleotides highlighted in red. The (identical) amino acid sequence deduced from both genes is indicated below the alignment.

Fig. S3. Map of plasmid pRJPaph-gfp_a1 derived from pRJPaph-gfp by replacing a PstI site downstream of GFP+ with AatII and KpnI served as scaffold for most of the analogous plasmids expressing alternative fluorescent proteins or the enzymatic marker GusA. Highlighted are the promoter Paph (blue), the GFP+ (green) and a 807 bp B. diazoefficiens DNA fragment originating from the downstream region of scoI (red; corresponding to B. diazoefficiens USDA110 genome position 1,245,228 to 1,246,034) which was used for chromosomal integration of pRJPaph-gfp_a1 and all derivatives via homologous recombination. Relevant restriction sites are indicated.
Figure 1
Ledermann et al. MPMI
Figure 2
Ledermann et al. MPMI
Figure 4
Ledermann et al. MPMI

284x375mm (300 x 300 DPI)
Supplemental Material

Construction of tagged *B. diazoefficiens* strains.

While construction of *B. diazoefficiens* strain GFP-1 is described in detail in the main body of this paper, cloning steps leading to the construction of all other tagged *B. diazoefficiens* strains are summarized here.

Using a (PstI)-AatII/KpnI adapter, the PstI site downstream of GFP+ on pRJPaph-gfp was replaced by AatII and KpnI resulting in pRJPaph-gfp_a1 (Fig. S3). The GFP+ gene in this plasmid was removed by BglII/KpnI digestion and replaced by compatible fragments comprising eBFP2, mTq2, or sYFP2 isolated from pGEM-PtuF-eBFP2, pGEM-PtuF-mTq2, and pGEM-PtuF-sYFP2, respectively (see Table S1). These plasmids were constructed by amplifying the promoter Ptuf from pMRE-105-mCherry and fusing it by overlap PCR to the genes of the respective fluorescent proteins. Source plasmids for the respective fluorescent proteins were pBAD-eBFP2 (a gift from Robert Campbell; Addgene plasmid # 14891), mTurquoise2-pBAD (a gift from Michael Davidson; Addgene plasmid # 54844) and pLM-sYFP2. Likewise, plasmid pRJPaph-HcRed was constructed by inserting HcRed as XbaI/PstI fragment (excised from the pTE100_HcRed methyllobrick vector (Schada von Borzyskowski et al., 2015) containing HcRed (Lambertsen et al., 2004); unpublished; kindly provided by R. Weishaupt and T. J. Erb, ETH Zürich, Switzerland) into SpeI/PstI-digested pRJPaph-gfp. To bring mCherry under the control of Paph, the gene was isolated as a HindIII fragment from pMRE-105-mCherry, cloned into pBluescript SK(+) to yield pBluescript-mChe_2 from where it was again released by SpeI/KpnI digestion and inserted into pRJPaph-gfp_a1.

Codon usage analysis of the GFP+ gene using JCat (Grote et al., 2005) and the *B. diazoefficiens* codon usage revealed a poor CAI value of 0.12. Accordingly, a GFP+ variant (bjGFP) which is codon-optimized for expression in *B. diazoefficiens* was chemically
synthesized (Eurofins Genomics, Ebersberg, Germany) and eventually brought under Paph control on plasmid pRJPaph-bjGFP. An alignment of the original GFP+ nucleotide sequence with that of the codon-optimized bjGFP gene is shown in Fig. S2

For construction of integrative plasmids expressing eBFP2, mTurquoise2, sYFP2, or mCherry from the Methylobacter extorquens promoter PtuF, the respective promoter fusions were excised as EcoRI-KpnI fragments from plasmids pGEM-PtuF-eBFP2, pGEM-PtuF-mTq2, pGEM-PtuF-sYFP2 and pMRE-105mChe and ligated with EcoRI-KpnI-digested pRJPaph-gfp_a1 to yield plasmids pRJPtuF-eBFP2, pRJPtuF-mTq2, pRJPtuF-sYFP2, and pRJPtuF-mChe.

For construction of B. diazoefficiens strain lacZYA-1, the pME3535_a2 derivative of pME3535 was generated by replacing the PstI site and the StuI site with SpeI and PstI, respectively.

Subsequently the promoter-less lacZYA-operon was excised from pME3535_a2 as a PstI-SpeI fragment and used to replace the gfp+ gene in pRJPaph-gfp. The resulting plasmid pRJPaph-lacZYA was integrated in the B. diazoefficiens chromosome downstream of scoI via homologous recombination.

For construction of B. diazoefficiens strain gusA-1, pGEM-PtuF-mTq2_a1 was created by AatII/KpnI digestion of pGEM-PtuF-mTq2, blunting and religation. The promoter-less gusA gene was excised from pCAM140 as an EcoRI-HindIII fragment and inserted into pGEM-PtuF-mTq2_a1 to create pGEM-gusAmTq2. In this plasmid, the HindIII site was replaced by an AatII site (pGEM-gusAmTq2_a1) and used as a source of gusA. The fragment was released by EcoRI-AatII digest and, after further modifications of the ends, used to replace the gfp+ gene in plasmid pRJPaph-gfp_a1. The resulting plasmid pRJPaph-gusA was integrated in the B. diazoefficiens chromosome downstream of scoI via homologous recombination.


**Table S1.** Complete list of strains and plasmids used in this work

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<td>pRJPtuF-mTq2</td>
<td>Tc&lt;sup&gt;+&lt;/sup&gt; (pRJPaph-gfp_a1) PtuF-mTurquoise2 for integration downstream of scoI</td>
<td>This work</td>
</tr>
<tr>
<td>pRJPtuF-sYFP2</td>
<td>Tc&lt;sup&gt;+&lt;/sup&gt; (pRJPaph-gfp_a1) PtuF-sYFP2 for integration downstream of scoI</td>
<td>This work</td>
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<tr>
<td>pRJPtuF-mChe</td>
<td>Tc&lt;sup&gt;+&lt;/sup&gt; (pRJPaph-gfp_a1) PtuF-mCherry for integration downstream of scoI</td>
<td>This work</td>
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<tr>
<td>pRJPaph-gusA</td>
<td>Tc&lt;sup&gt;+&lt;/sup&gt; (pRJPaph-gfp_a1) Paph-gusA for integration downstream of scoI</td>
<td>This work</td>
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<tr>
<td>pRJPaph-lacZYA</td>
<td>Tc&lt;sup&gt;+&lt;/sup&gt; (pRJPaph-gfp) Paph-lacZYA for integration downstream of scoI</td>
<td>This work</td>
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Table S1. Ledermann et al., MPMI

<table>
<thead>
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<th>Name</th>
<th>Title</th>
<th>Journal</th>
<th>Pages</th>
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Table S2. Oligonucleotides used in this study

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<th>Oligo</th>
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<tr>
<td>(PstI)-AatII/KpnI</td>
<td>AGGTACCGACGTCTTTGCA</td>
<td>Replacement of PstI restriction enzyme site in pRJPaph-gfp with</td>
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<td>AGACGTGGATACCTTTGCA</td>
<td>AatII and KpnI sites to construct pRJPaph-gfp_a1</td>
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<tr>
<td>(HindIII)-AatII</td>
<td>AGCTGACGTC</td>
<td>Replacement of HindIII restriction enzyme site in pGEM-gusAmTq2 with</td>
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<td>an AatII site to construct pGEM-gusAmTq2_a1, self-anealing</td>
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<tr>
<td>(PstI)-SpeI</td>
<td>CACTAGTGCTGCA</td>
<td>Replacement of PstI restriction enzyme site in pME3535 with an SpeI</td>
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<td>site to construct pME3535_a2, self-anealing</td>
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<tr>
<td>(Sfil)-PstI</td>
<td>GAACTGCAAGAAG</td>
<td>Replacement of Sfil restriction enzyme site in pME3535 with an PstI</td>
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<td>CTTCCTGAGTTC</td>
<td>site to construct pME3535_a2</td>
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<td>PtuF_fwd_NotI</td>
<td>GACGCGCGCGGCTAGATGACATGCTGAGGC</td>
<td>Primer for amplification of tuF promoter</td>
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<tr>
<td>PtuF_rev</td>
<td>CTGCCTTTGGCTCACCAT</td>
<td>Primer for amplification of tuF promoter</td>
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<td>XFPs_for</td>
<td>ATGGTGAGCAAGGCGGCGAG</td>
<td>Primer for amplification of eBFP2, mTurquoise2 and sYFP2</td>
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<tr>
<td>XFPs_rev_KpnI</td>
<td>CAGGGTACCTTACTTGTACAGCTGAGTC</td>
<td>Primer for amplification of eBFP2, mTurquoise2 and sYFP2</td>
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<td>xxFP-1</td>
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<td>Forward primer for verification of correct integration of eBFP2,</td>
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<td>mTurquoise2 and sYFP2 in the chromosome</td>
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<td>Forward primer for verification of correct integration of GFP+ into</td>
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<td>mChe-1</td>
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<td>HcRed-1</td>
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<td>lacA-1</td>
<td>TAAACGAGCAGGATAAGCA</td>
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<td>gusA-3</td>
<td>AGCAGGGAGGCAAACACG</td>
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<td>gfp+4</td>
<td>AGATATTCGCGGCGGATTTC</td>
<td>Reverse primer for verification of correct integration of plasmids into scoI downstream region in chromosome, binds to chromosomal region</td>
</tr>
</tbody>
</table>
Figure S1
Ledermann et al. MPMI
Figure S3
Ledermann et al. MPMI