Systematic and Applied Microbiology 38 (2015) 515–523

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Symbiotic effectiveness and phylogeny of rhizobia isolated from faba bean (*Vicia faba* L.) in Sichuan hilly areas, China

Kai Wei Xu[a,*,1], Lan Zou[a,1], Petri Penttinen[b,1], Ke Wang[a], Nan Nan Heng[a], Xiao Ping Zhang[a], Qiang Chen[a], Ke Zhao[a], Yuan Xue Chen[a,**]

[a] Department of Microbiology, College of Resources, Sichuan Agricultural University, Chengdu, 611130, China
[b] Department of Environmental Sciences, University of Helsinki, Helsinki, Fin-00014, Finland

ARTICLE INFO

Article history:
Received 16 April 2015
Received in revised form 19 June 2015
Accepted 22 June 2015

Keywords:
Faba bean ;·Symbiotic effectiveness
;·Genetic diversity ;·MLSA
Rhizobia

ABSTRACT

A total of 54 rhizobial strains were isolated from faba bean root nodules in 21 counties of Sichuan hilly areas in China, and their symbiotic effectiveness, genetic diversity and phylogeny were assessed. Only six strains increased the shoot dry mass of the host plant significantly ($P \leq 0.05$). Based on the cluster analysis of combined 16S rDNA and intergenic spacer region (IGS) PCR-RFLP, the strains were divided into 31 genotypes in 11 groups, indicating a high degree of genetic diversity among the strains. The sequence analysis of three housekeeping genes (*atpD*, *glnII* and *recA*) and 16S rDNA indicated that the strains represented two *R. leguminosarum*, two *Rhizobium* spp., *R. mesosinicum*, *Agrobacterium* sp. and *A. tumefaciens*. The strains representing four *Rhizobium* species were divided into two distinct *nodC* and *nifH* genotypes. However, the phylogeny of housekeeping genes and symbiotic genes was not congruent, implying that the strains had been shaped by vertical evolution of the housekeeping genes and lateral evolution of the symbiotic genes.

Introduction

Symbiotic biological nitrogen fixation by legume – rhizobium symbiosis is a key component in sustainable agriculture, where nitrogen fixation takes place in root or stem nodules formed by the legume and its rhizobial symbiont. Formation of nodules requires compatible partners. Selective legumes form nodules together with only a few rhizobial strains, whereas promiscuous legumes are nodulated by a wide range of rhizobia.

Faba bean (*Vicia faba* L.), a grain legume with a high protein and starch content, is grown worldwide for food and feed [17,42]. Some faba bean varieties produce L-DOPA (3,4-dihydroxyphenylalanine) that has medical applications [9,38]. In 2013, the world production of faba bean was 3.5 million tons, of which 1.4 million tons was produced in China [11]. Among the grain legumes, the quantity of nitrogen fixed by faba bean – rhizobium symbiosis is one of the highest, reaching up to 200 kg N ha^{-1} per year [16]. In addition, intercrop faba bean increases the yield and phosphorus use efficiency [24,45,51].

Faba bean is considered to be a selective host that is commonly nodulated by *Rhizobium leguminosarum* symbiovars viciae, trifolii and phaseoli, *R. etli* and *R. fabae* [35,36,38]. The other symbionts include *R. laguerreae* [31], *Agrobacterium tumefaciens* and *Agrobacterium* spp. [46]. In legume – rhizobium symbiosis, the composition of the nodulating population varies, which is mostly explained by differences in soil condition, climate and plant variety [1,4,8,19,39]. In addition, the nodulating strains show diverse nitrogen fixation efficacies varying from highly efficient to inefficient and even parasitic [25,43]. The faba bean varieties may be divided into two distinct groups [9]. The spring faba beans are cultivated in temperate climates, for example in Europe and Northern China. The winter faba beans are grown in areas with a mild or sub-tropical climate, for example, in the Mediterranean and Southern China. Based on the sequence of the symbiosis related gene *nodD*, the faba bean symbionts may be divided into groups that roughly correspond to the division of spring and winter faba beans [37].

The hilly areas in the center of Sichuan basin with a subtropical wet monsoon climate are the main faba bean production areas in southeast China. The soil in the area is diverse, with the main types

* Tel.: +86 28 8629 0982; fax: +86 28 8629 0983.
** Tel.: +86 28 8629 1179; fax: +86 28 8629 0983.
E-mail addresses: xkwei@sicau.edu.cn (K.W. Xu), 18200353226@163.com (L. Zou), petri.penttinen@helsinki.fi (P. Penttinen), 1173704119@qq.com (K. Wang), 727805451@qq.com (N.N. Heng), zhangxiaopingphd@126.com (X.P. Zhang), cqiang@sicau.edu.cn (Q. Chen), zhaoke82@126.com (K. Zhao), cyxue2002@aliyun.com (Y.X. Chen).
[1] These three authors contributed equally to this work.

http://dx.doi.org/10.1016/j.syapm.2015.06.009
0723-2020/

being purple soil, paddy soil, and yellow soil, which have a pH range of 3.1–8.8 [7]. In Sichuan, more than 200 locally bred faba bean winter-type varieties are cultivated as the sole crop or are intercropped with maize or wheat [10,33,48–50]. The diversity of soil conditions and host plants is expected to be accompanied by rich genetic diversity of rhizobia. To our knowledge, prior to this study, there were no systematic studies on the genetic diversity and phylogeny of faba bean nodulating rhizobia in Sichuan. Therefore, the objectives of this study were to find effective nitrogen fixing potential inoculant strains compatible with faba bean, and to investigate the genetic diversity and phylogeny of faba bean rhizobia in this area.

Materials and methods

Isolation of rhizobia

Nodules were collected from faba bean roots (*Vicia faba* L.) in 21 counties in Sichuan hilly areas, China (Table 1). Bacteria were isolated from surface sterilized nodules as described by Xu et al. [43] on yeast – mannitol agar (YMA) medium [40] containing 25 mg mL^{-1} Congo red at 28 °C. The pure isolates were saved on YMA slants at 4 °C for temporary storage and in 25% glycerol at −80 °C for long-term storage.

Nodulation and symbiotic efficacy test

The nodulation and symbiotic efficacy test was conducted as described previously [43], except that the seeds of faba bean variety 'Dabaidou' were soaked overnight in sterilized water after surface sterilization because of the thick, hard seed coat. After 50 days, the plants were harvested, and the shoot dry weights and numbers of nodules were determined. Excel 2010 (Microsoft, Redmond, USA) and SPSS 17.0 (SPSS Inc., Chicago, USA) were used to calculate one-way analysis of variance with a least significant difference (LSD) analysis ($P \leq 0.05$) of the main values.

DNA extraction and PCR-RFLP

DNA was extracted as described by Little [21]. 16S rDNA was amplified using 20 pmol primers P1 (5′-AGA GTT GAT CCT GGC TCA GAA CGA ACG CT-3′) and P6 (5′-TAC GGC TAC CTG TAC GAC TTC ACC CC-3′). The intergenic spacer region (IGS) was amplified using 20 pmol primers pHr (F) (5′-TGC GGC TGG ATC ACC TCC TT-3′) and p23SR01(R) (5′-GGC TGC TTC TAA GCC AAC-3′). The amplifications were carried out in a Bio-RAD MyCycler™ using the Golden Easy PCR System (TIANGEN, Beijing, China) and 50 ng of DNA as template, as described by Xu et al. [43] except that the annealing temperature was 60 °C for IGS.

Aliquots (5 μL) of the PCR products were digested by the restriction endonucleases *Msp*I, *Hae*III, *Hin*fI and *Taq*I separately in a 10 μL reacting volume following the manufacturer's instructions (Fermentas, EU). The restriction fragments were separated by electrophoresis in 2% agarose gel at 80 V for 3 h, thereafter stained with ethidium bromide (0.5 μg mL^{-1}) and photographed with the Gel Document System. The RFLP analyses of 16S rDNA (ARDRA) [38] and the IGS, and cluster analysis of combined ARDRA and IGS-RFLP (CACAI) [15], were conducted using the UPGMA clustering algorithm in the NTSYSpc program [30].

Multilocus sequence analysis

Based on CACAI and symbiotic performance, representative strains were selected for sequencing of 16S rDNA, *atpD*, *recA*, *glnII*, *nodC* and *nifH* genes. The 16S rDNA fragments were amplified as described above for 16S rDNA PCR-RFLP. The *atpD*, *recA*, *glnII*, *nodC* and *nifH* genes were amplified using primer pairs atpD255F/atpD782R [15], glnII12F/glnII689R [41], recA41F/recA640R [41] nodCF/nodCI [32] and nifHFctg/nifHR [14], as described in the respective references. The amplified fragments were directly sequenced at BGI Tech (Shenzhen, China). The sequences of the faba bean isolates and reference sequences were aligned with ClustalW in MEGA 6.0 [34]. Phylogenetic trees were constructed using the neighbor-joining and maximum likelihood methods, and the confidence level was determined by 1000 replicates and bootstrapping in MEGA 6.0 [34]. Concatenated sequences of the three housekeeping genes (*atpD*, *recA*, and *glnII*) were analyzed for multilocus sequence analysis (MLSA) by applying 97% similarity as the threshold for defining genospecies [4,28].

Results

Isolation and symbiotic efficacy of faba bean-nodulating rhizobia

Rhizobia were isolated from root nodules collected from faba bean growing in 21 counties in Sichuan hilly areas, China. Out of the 54 isolates, 48 formed nodules on faba bean roots. The average nodule numbers ranged from 2.3 to 184.6 per plant (Table 1). Compared to the uninoculated control, six isolates increased the shoot dry mass significantly ($P \leq 0.05$) and were considered good candidates as inoculants for faba bean (Table 1). However, unexpectedly, nodulation by strain SCAUf32 resulted in a statistically significant decrease in shoot dry mass.

ARDRA, IGS-RFLP and CACAI analyses

In the 16S rDNA PCR, an almost 1500-bp fragment was amplified. In the 16S rDNA PCR-RFLP, 51 out of the 54 strains clustered together as the type A genotype, while only one strain was found in each of types B, C, and D, respectively (Table 1). Most of the strains produced one band in the IGS PCR, whereas a few strains produced two bands, and the lengths of the bands ranged from 1900 bp to 2200 bp. Altogether, five IGS PCR types were obtained (Table 1). The IGS-RFLP showed more variation than 16S rDNA RFLP, resulting in 31 different IGS-RFLP genotypes. Based on the combined analysis of ARDRA and IGS-RFLP (CACAI), 31 genotypes were distinguished and 11 groups were classified at the 93% similarity level (Fig. 1). Out of the six significant growth promoting strains, two were in CACAI group I, three in group IV and one in group VI (Figs. 1, S1).

Phylogenetic analyses of housekeeping genes and MLSA

Based on CACAI and symbiotic performance, 12 representative strains were selected for sequencing of three housekeeping genes, 16S rDNA and two symbiosis related genes. The three housekeeping genes (*atpD*, *glnII* and *recA*) were sequenced from all the representative strains. The phylogenetic trees based on the neighbor-joining method were congruent with those based on the maximum likelihood method (Figs. 2–5 , S2–S9). Based on both the single gene trees and MLSA, seven strains from six CACAI groups clustered into two distinct subgroups related to *R. leguminosarum* with similarities from 98.7% to 100% (Figs. 2, S4–S9). The strains were assigned as *R. leguminosarum* I and *R. leguminosarum* II. Strain SCAUf15 clustered with *R. leguminosarum* with similarities from 95.8% to 99.2% in the single gene trees and 96.7% in the MLSA. Thus, it was assigned to *Rhizobium* sp. I. SCAUf54 clustered separately in both the MLSA and single gene trees with similarities of <94.2%, and it was assigned to *Rhizobium* sp. II. SCAUf20 clustered with *R. mesosinicum* with 98.7% similarity in the MLSA tree and the clusters in the single gene trees were congruent with that of MLSA. Therefore, SCAUf20 was assigned as an *R. mesosinicum* strain. SCAUf21