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Application of Green Fluorescent Protein Gene (*gfp*) in the Symbiosis between *Mesorhizobium Huakuii* and *Astragalus Sinicus*

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Green fluorescent protein (GFP) is a luminescent protein which was first discovered from *Aequorea victoria*[1]. The chromophore of wild type GFP consists of an imidazolone ring formed by cyclization of Ser65, dehydrated Tyr66 and Gly67. GFP can emit 510 nm green fluorescence (Emmax = 510 nm) by 395 nm violet (Exmax=395) or 475 nm blue excitation[2]. GFP has been used as a new report gene with following priorities: It is a 28 KD protein of 238 amino acids, no toxic effect on cell growth, no interference with the function and localization of marked proteins, can be studied under many situations for its stable molecular structure and light emission mechanism. Additionally, GFP is the only luminescent protein which can express in heterologous cells. It can be monitored within living cells since its fluorescence is independent of any substrate. Up to now, it has been accepted that GFP is one of the best methods to study gene expression and protein localization within living cells which shows a broader use in future. Since Chalfie et al first expressed GFP in *Caenorhabbititis elegans* and *Escherichia coli* in 1994 [3], it has been used in studies of many organisms species such as bacteria, anabaena, myxobacteria, yeasts, plants and animals. For its non species-specific character, GFP has been wildly used as fluorescent marker especially in monitoring gene expression and protein localization in living cells.

Astragalus sinicus is one of legumes mainly cultivated as an important green manure and nectar source in the south of China. *Mesorhizobium huakuii* can infect *A. sinicus* to form nitrogen-fixing nodules, and belong to a unique cross-inoculation group[4,5]. The symbiosis between *M.huakuii* and *A.sinicus* is a China-specific resource. Comparing with other model *rhizobium*, however, the studies on gene localization, structure and function during the early nodulation stages of *M.huakuii* were much backward. Our research group has conducted studies on the metabolism, physiology, biochemistry, cell differentiation during symbiotic development and taxonomy of *M.huakuii*. We have also studied the early symbiosis between *M.huakuii* and *A.sinicus* by using *gfp* as marker gene in recent years which was founded by the National Natural Science Foundation. The main research works included: to confirm *gfp* heterlogous expression in *M.huakuii*, to study conditions and problems during its expression, to construct *gfp*-based broad-host-range promoter probe vector, to trap the constitutive promoters and specific promoters induced by *A.sinicus* seed extract, and to monitor *M.huakuii* during the early events of nodulation process.

1 Construction of Promoter -probe Vector with Wild type *gfp* as Reporter Gene

1.1 Construction and confirmation of broad-host-range promoter probe vector pHN127 with wild type *gfp* as reporter gene

A recombinant plasmid pHN115 was constructed by ligation of a fragment containing *gfp* gene into the expression vector pET11-C. An 1 kb *Eco*RI-*Xba*I fragment from pHN115 was ligated into pIJ2925 for the construction of plasmid pHN117, in which a Shine-Dalgarno sequence was kept in upstream region of the promoterless *gfp* gene. The restriction sites of *Xba*I, *Bam*HI, *Sma*I, *Kpn*I and *Sac*I could be used to clone promoters. The 1 kb *Bgl*II fragment from pHN117 was inserted into a broad-host-range vector pTR102 digested with *Bam*HI to create promoter probe vector pHN127. To confirm its ability for promoter trapping, the 665 bp fragment contained a *tet*A/*tet*R bidirectional promoter from pBR322

digested with *Sau*3AI was cloned into *Bam*HI-digested and alkaline phosphatase-treated pHN127. Transformants of *E.coli* DH5 α was grown at 28℃ and colonies was detected for fluorescence under long-wave violet. Results of all colonies emitting green fluorescence sho wed the promoters trapping ability of pHN127.

1.2 Trapping constitutive promoter fragments of *M. huakuii* 7653R

Total DNA extracted from *M.huakuii* 7653R was partially digested with *Sau*3AI and then ligated to *Bam*I-degested and alkaline phosphatase-treated pHN127.The ligation mixture was transformed into *E.coli* DH5 α. Transformants were grown at 28℃. Colonies emitting green fluorescence could be screened under long-wave violet. The plasmid in the colony with brightest fluorescence was isolated and named pHN136. The restriction map of pHN136 was confirmed that it contained the transcription-constitutive regulative DNA fragment.

1.3 In situ monitoring of wt*gfp* expression in *M. huakuii* under symbiotic conditions

pHN136, pHN129 and pHN127 were introduced individually into *M.huakuii* 7653R. The transformants were used to inoculate using self-designed mini-root-box apparatus *A.sinicus* and monitored in situ during the early nodulation process with a fluorescent microscope. Because of the interference of plant autofluorescence, it was difficult to detect the expectant infection process. Fluorescence was also not detected in or from whole nodules which the microaerobiosis in nodules might not be favorable for GFP's maturation. But the rhizobia isolated from nodules could emit green fluorescence when by long-wave violet As indicated above pHN136 was stable in *M.huakuii* under symbiotic conditions.

It was found that the *gfp* gene in pHN127 could only be effective for the promoters with stronger transcriptional activity but not the weakers so that it was difficult to trap induced promoters. To broaden the application field, new GFP mutants with enhanced fluorescent sensitivity and soluble ability was used for next experiments.

2 Construction and Application of the Broad-host-range Promoter Probe Vector pHN1006 Based on *gfp*mut3

Cormark et al replaced the sequence region encoding the chromophore Ser-Tyr-Gly and the flanking 55-77 amino acids by site-directed mutagenesis. The mutated *gfp* library controlled by strict inducible promoter Ptac was constructed in *E.coli*[6]. Three mutant proteins EGFPmut1, 2 and 3 with fluorescent intensity 20-35 fold higher than wtGFP were screened by the technique of fluorescence-activated cell sorting (FACS). Among the three mutants, EGFPmut3 (S65G, S72A) could express well in bacteria with excitation and emission maxima of 501 nm and 511 nm, respectively. For the detection in a single cell, EGFPmut3 might be the best choice for *gfp* expression in bacteria.

2.1 Construction of broad -host -range promoter probe vector pHN1006

The promoter probe vector pHN1005 in *E.coli* was constructed. The *Bgl*II digested 1.7 kb fragment containing promoter-probe unit from pHN1005 was ligated to pTR102 which was *Bam*HI-digested and alkaline phosphatase-treated. The broad-host-range promoter probe vector pHN1006 based on *gfp*mut3 was obtained and its characters was the following:

(1) The *Bam*HI site at 5'-end upstream of *gfp*mut3 could be used to clone DNA fragments with promoter-active and quantitativelyes analysis transcriptional activities of inserted promoter.

(2)The rRNA T_1T_2 terminator at 3'-end downstream of *gfp*mut3 permitted to clone strong active promoters.

(3)Another rRNA T_1T_2 terminator was also inserted upstream of *Bam*HI site to avoid transcription from the promoter on the vector pUC19 background.

(4)An intron fragment was just inserted upstream of *gfp*mut3 codon region to stop the translation of all six reading frames in both directions for ensuring the proper translation of $gfpmut_3$. In addition, the sites of *Sma*I, *Kpn*I and *Sac*I could be used to identify the promoter fragment.

2.2 Cloning promoter -active fragment from the genome of *M.huakuii* 7653R

Genomic DNA isolated from *M.huakuii* 7653R was partially digested by Sau3AI and ligated to *Bam*HI-digested and alkaline phosphatase-treated pHN1006. The ligation mixture was introduced into 7653R competent cells with electroporation. Transformants were selected on TY agar containing Ap. When illuminated under long-wave violet, some transformants

appeared green fluorescence while the fluorescence of control 7653R (pHN1006) could not be detected. Results of diverse fluorescent colonies proved that the constitutive promoters with different activities were obtained. The plasmid of the brightest transformant was extracted and named pHN2-33. The transformants showing no or weak fluorescence were inoculated on TY+AP+ seed-extract from *A.sinicus* and TY+Ap plates respectively. Among ten thousand colonies, a recombinant emitting magnificent fluorescence with the induction of *A.sinicus* seed extract while no fluorescence without such induction was screened. The plasmid isolated was named as pIN32. It was again electroporated into 7653R to confirm the fluorescent phenotype. The transformant was renamed as IN32.

In this study, a broad-host-range promoter probe vector pHN1006 with *gfp*mut3 as reporter gene was constructed. The screening of a series recombinants with different fluorescent intensities showed that promoter-active DNA fragments were cloned. The differences in fluorescent intensities indicated the diversities of *gfp* expression at the transriptional level. A recombinant emitting strong fluorescence with the induction of *A.sinicus* seed extract was also obtained. These results provided a new idea for cloning more symbiosis-specific genes and also strains for further study on molecular genetics of *M.huakuii*.

3 In situ Monitoring Marked *M.huakuii* 2-33 during the Early Nodulation Stage

Strain *M.huakuii* 2-33 with expression of *gfp*mut3 was used to monitor in situ cell development and differentiation during the early nodulation process.

3.1 The stability of marker plasmid and the effect of GFP expression on physiology, metabolism and symbiotic property of rhizobia

Colonies of continuous reinoculation on nonselective plates for eight times were randomly selected and illuminated under long-wave violet. No difference in fluorescent intensities was observed. The stable fluorescent ratio of colonies calculated during inoculations showed that the marker plasmid was very stable in host rhizobia under free-living conditions.

A.sinicus growing in mini-root-box was inoculated with 2-33 and control 7653R. Strong fluorescence from early nodules and 20-day-old mature nodules could be detected with the fluorescent microscope. The rhizobia isolated from these nodules could also form colonies showing green fluorescence under UV light. These results proved that the marker plasmid was stable during symbiosis. The nodules were also examined using transmission electron microscope and scanning electron microscope. Results demonstrated that the supermicro-structures of nodules, bacteroid-containing cells and bacteroids of 7653R and 2-33 were similar.

The growth curves of 2-33 and 7653R were determined. No significant difference was found. The above results showed that the existence and expression of *gfp*-mut3 had no significant effect on host cells.

3.2 In situ monitoring rhizobia during the early nodulation process of *M.huakuii* –*A.sinicus* symbiosis

A.sinicus was inoculated with 2-33 in mini-root-box. Two days later, the marker strain 2-33 was monitored with laser confocal scanning microscope (LCSM) to research its root colonization, root hairs infection and green fluorescence of GFP expression in different layers of mature nodules.

In spite of autofluorescence of *A.sinicus* roots, the GFP expression could be well detected under LCSM. In different layers of the nodule, only bacteroid-containing cells could produce fluorescent signal which confirmed that fluorescence originated really from *gfp* marked bacteroids.

The fluorescent signals from rhizobia, bacteroids and nodules were recorded and analyzed using Zeiss fluorescent microscope equipped with Charged Coupled Device (CCD) lens and FISH program. The bacteroids under fluorescent microscope showed diverse forms such as pear, Y, spindle and irregular shapes, which were corresponding to the results detected by electron microscope.

4 Expression of Blue Fluorescent Protein (BFP) Gene in *M.huakuii*

Heim et al randomly mutated wt*gfp* with error-prone PCR and screened mutants with spectra altered under the excitation of 475 nm and 395 nm UV light[7]. One mutant gene Y66H P4 producing bright blue fluorescence under near UV light was named as *bfp* gene. The *bfp* gene was subcloned and successfully expressed in *M.huakuii* 7653R. These results gave the pos-

sibility for the expression regulation of different genes which could be monitored in situ when marked with *gfp* and *bfp*. With the technique of fluorescent resonance of energy transfer (FRET), the subcellular localization of different proteins in the interaction between rhizobia and host plants could also be detected.

5 Conclusion

A set of vectors and methods based on *gfp* genes to study the molecular genetics during the early nodulation process of rhizobia-legume symbiosis were established in this work. By using the strict promoter probe vector based on gfpmut3, some constitutive promoter fragments and a inducible promoter from *M.huakuii* were obtained. All the results provided strains for further study on molecular biology of *M.huakuii* and offered a new method to study gene expression regulation in the symbiosis between rhizobia and legume plants.

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