Transformation Studies of *Bacillus thuringiensis cryIC* Gene into a Nitrogen-Fixing *Azospirillum lipoferum*

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A lepidopteran toxin gene, *cryIC* (pSB607) from entomopathogenic *Bacillus thuringiensis* subsp. *aizawai* was introduced into nitrogen-fixing *Azospirillum lipoferum* by transformation. Regeneration of spheroplasts was achieved at 99% with 39% frequency of regeneration. Transformants were screened on NB kanamycin with ampicillin plates and 4 transformants were selected after ten generations. SDS-PAGE and Western blot analysis confirmed the presence of a 68 kDa protein in the transformants. Studies on utilization of carbon sources indicate that glucose and sucrose are the most favorable carbon sources and 2% molasses is the cheap alternate carbon source for the better growth of parent *A. lipoferum* and transformants.

Introduction

Many C_4 plants such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), sugar cane (*Saccharam* sp.) sorghum (*Sorghum bicolor*), maize (*Zea mays*), *Panicum maximum*, *Pennisetum purpureum* and forage grasses such as *Brachiaria humidicola*, *Leptochola fusca* are colonized by *Azosprillum* (James *et al.*, 1998). Some plants have chemotactic attraction towards *Azospirillum* (Rovira, 1970), where it associates as non-specific, beneficial bacteria and enhances the growth of some non-symbiotic plants such as tomato (Bashan *et al.*, 1989), soybean and seedlings of cactus, *Pachycereus pringlei* (Purnte *et al.*, 1993). It fixes nitrogen in association with the roots of different C_4 plants (Dübereiner and Pedrosa, 1987) and increases the crop yields.

The soil-dwelling *Azospirillum* commonly associated with cortical root cells, intercellular spaces of roots of symbiotic plants (James *et al.*, 1998), however it also found in stems, leaf sheaths, and leaves of several field and *in-vitro* grown plants especially stems of graminaceous species and in the leaves of finger millet (*Eleusine coracana*) and *Setaria italica* (Agarwala *et al.*, 1988). Cereals like corn and wheat can harbor about 10^4–10^7 cells/g dry weight (Dübereiner and Pedrosa, 1987).

The potential for widespread use of *Bacillus thuringiensis* (Bt) based on microbial formulations is timely, because of their relative host specificity, ecological non-disruptive and degradable nature. Application of Bt has been most successful, when it is delivered as foliar sprays against lepidopteran pests such as *Spodoptera litura*, *Heliothis armigera*, *Trichoplusia ni* etc (Rajendran and Venkatesan, 1993). Similarly, expression of a high level of insecticidal crystal protein (ICP) in plants showed a high level of protection against some of the major economic pests such as *Plodia interpunctella* of maize (Giles *et al.*, 2000), Spanish corn borer (*Sesamia nonagrioides*) of Spanish corn, European corn borer (*Ostrinia nubilalis*) of corn hybrids (Gonzalez *et al.*, 2000), yellow stem borer of indica rice (Alam *et al.*, 1998), fruit borer (*Helicoverpa armigera*) of tomato (Mandaokar *et al.*, 2000) and five major pests of soybean (*Glycine max*) such as corn ear worn, verlvet-bean caterpillar, *Helicoverpa zea*, *Anticarsia gemmatalis* and *Elasmopalpus lignosellus* (Walker *et al.*, 2000). However the lack of agronomic performance of Bt and non-Bt plants in the absence of pests, redesigned the integrated pest management (IPM) strategy towards a synergistic approach.

In many plants such as soybean, sorghum and rice the yield performance can be increased with

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the incorporation of growth promoting bacteria such as *Azosprillum*. Similarly, expression of ICP gene in *Azosprillum* and further amplification of soluble ICP inside the plant tissues could accumulate a higher soluble protein as protoxin (Mcbridge *et al.*, 1995) to facilitate their capacity of inducing feeding deterrence or mortality at lower dosage levels against economic pests. Hence, the synergistic delivery of plant growth promotion and plant protection against target pests would be an added advantage for crop improvement as reported in *Pseudomonas fluorescens* (Rajendran *et al.*, 1994). In order to achieve such a synergistic delivery, a transformation method was attempted for an integration of *cryIC* gene of *B. thuringiensis* subsp. *aizawai* into *A. lipoferum*.

Materials and Methods

Azospirillum lipoferum, a laboratory stock was maintained in semi-solid nitrogen-free basal medium (NFb) as described by Dübereiner and Day (1987). The NFb with 1 g/l NH_4Cl plus KNO_3 was used for the aerobic growth at 28 °C for 24–36 hrs at 180 rpm. The NK broth (nutrient broth with KNO_3 4 g/l) was used for regeneration of spheroplasts. In order to replace malic acid as carbon source in the rich NFb medium and to study the utilization of the most favorable carbon source by *A. lipoferum*, glucose, maltose, sucrose, lactose and mannitol were examined. As a cheap alternative carbon source, molasses (MO) was tried at various concentrations. Filter paper sensi-discs were used for antibiotic resistance.

The Tris-sucrose-EDTA-lysozyme method described by Weiss (1976) was followed to isolate the spheroplast. In this method, cells harvested by centrifugation from NFb medium were resuspended in 100 mM Tris-HCl [Tris (hydroxymethyl) aminomethane-hydrochloride] buffer (pH 8.0) containing 0.5 M sucrose, washed twice, and finally dissolved in the same buffer. Cell density in the buffer was then adjusted to have viable cell counts between 10^8 to 10^9 cell/ml. A volume of 1 ml of 100 mM EDTA (disodium salt) was added with 9 ml of cell suspension drop by drop and incubated for 1 hour. Crystalline lysozyme (10 mg/ml) was then added to the suspension, mixed gently and further incubated for 24 hours. The spheroplasts formed were centrifuged twice, after suspending in hypertonic sucrose NFb medium. The pellet was finally dissolved in 9 ml of the hypertonic sucrose medium. Dilutions of the suspension were made in the respective hypertonic sucrose medium for counting spheroplasts as well as in distilled water for counting cells after osmotic cell lysis. Plasmid isolation, SDS-PAGE, immunoblot (Western) analysis were made as described by Sambrook *et al.*, 1989.

Transformation of *A. lipoforum* was carried out by a modification of the $CaCl_2$ method as reported for *Pseudomonas* (Sano and Kagayawa, 1977). In brief, the cell pellet of *A. lipoferum* recovered at ± 0.3 OD, was suspended with 5 ml of 100 mM $MgCl_2$ (resting for 15 minute), after centrifugation a 5 ml of 30 mM $CaCl_2$ was added to the pellet then resting for 60 minute and centrifuged. The pellet was dissolved with 200 μl of 30 mM $CaCl_2$ (resting for 60 minute). Pre-chilled condition was followed in every step. To each 80 μl of this cell suspension, 10 μl of (0.4 μg) pSB607 plasmid DNA (a gift from Dr. Donald Dean, Ohio State University, USA) suspended in TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA) was added and kept on ice with intermittent swirling. This was heat-shocked at 42 °C for 5 minute, cooled to 0 °C for 2 minute, then immediately added with 1 ml of NK broth and kept on shaker at 37 °C for 2 hours. Screening and selection of transformants were made on NB Kan^r with Amp^r plates after 16 hours of incubation at 37 °C.

Results and Discussion

In recent years, much attention has been focused on the molecular aspects of *Azospirillum* in order to utilize the microbe as biofertilizer. Existence of a notable relationship between nitrogen fixation by *Azospirillum* sp. and certain organic acids such as malate and sugars, supplied in nature by host plants has been noticed. These compounds occur in C_4 plants as root-exudates exert a chemotactic effect towards *Azospirillum* (Rovira, 1970). In the present study on the most favorable carbon sources indicates that glucose and sucrose were effectively utilized by *A. lipoferum* and helps better growth (Fig. 1A). Out of cheap alternative-carbon sources, 2% (w/v) molasses supported better growth than the other molasses fractions studied (Fig. 1B). The biomass enhancement study indi-

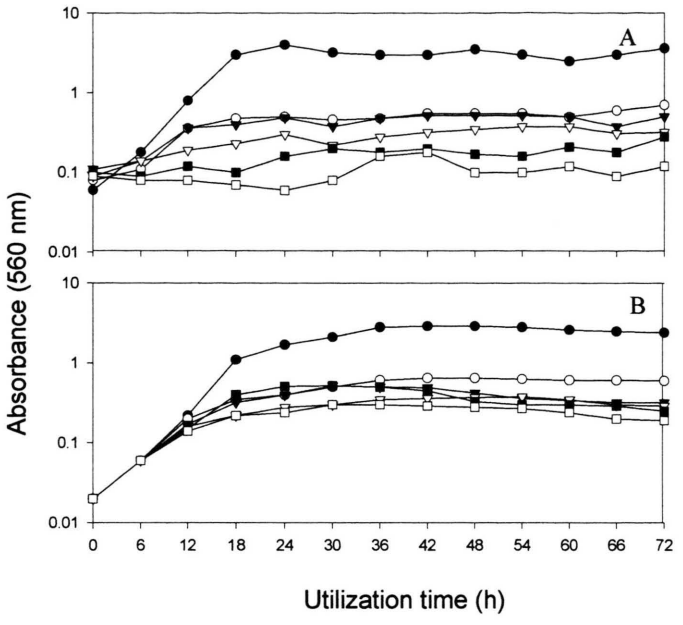

Fig. 1. Growth pattern of *Azospirillum lipoferum*. Utilization of most favorable carbon sources by *A. lipoferum* was shown in the upper part of the figure (A) using various carbon sources such as sucrose (○), glucose (▼), maltose (▽), lactose (■) and mannitol (□). LB medium (●) was used as control. Out of the five tested carbon sources, sucrose and glucose were effectively utilized by the test organism by indicating better growth throughout the growth period. Mannitol was poorly utilized, when it was given as sole carbon source.
Utilization of cheap alternative carbon source by *A. lipoferum* was shown in the lower part of the figure (B) using various percentage of molasses (W/V) as sole carbon sources such as 1% molasses with NaCl (▼), 2% molasses with NaCl (○), 3% molasses with NaCl (■), 4% molasses with NaCl (□) and 1% molasses without NaCl (▽). LB medium (●) was used as control. Out of the five tested concentrations of molasses, 2% molasses with NaCl was effectively utilized by indicating better growth throughout the growth period.

cates that addition of 0.5% of yeast extract yielded maximum cell density in contrast to all other molasses combinations tried. This could be due to the presence of certain essential nutrients and organic acids in the yeast extract. Similar growth patterns were observed in all four transformants (data not shown) using both favorable and cheap alternative carbon sources. It indicates that no impairment occurred in the metabolic growth activities of the transformants of the *A. lipoferum* due to the transformation of the plasmid pSB607, carrying the *cryIC* gene.

In an earlier study, expression of plasmid pRK290 was achieved through a transformation in *A. brasilense* (Fani *et al.*, 1986). The present study attempts to transform a plasmid pSB607, carrying the *cryIC* gene into spheroplasts of *A. lipoferum*. The antibiotic assay studies indicates that *A. lipoferum* showed resistance to kanamycin and rifampicin at a concentration of 60 and 30 μg/ml respectively, while it was sensitive to chloramphenicol, streptomycin, ampicillin and tetracycline. Initially we were unable to regenerate the spheroplast of *A. lipoferum,* after transformation of the target plasmid. However, it was achieved after several trials, using modification in the existing transformation protocol of *A. brasilense* by Sano and Kagayawa (1977). The efficiency of transformation ranged from 500 to 750 transformants per μg of plasmid DNA. Colonies were rescreened on antibiotic plates for ten generations and four prominent transformants were selected after loosing most of them in the stability-checking regeneration process on double antibiotic plates.

In the present study, the regeneration of spheroplasts in *A. lipoferum* was achieved to 99% and

Table I. Frequency of spheroplasts formation and regeneration in *Azospirillum lipoferum.*

Strains	Initial cell count (ml^{-1}) A	Viable cells after osmotic shock (ml^{-1}) B	Viable cells after regeneration (ml^{-1}) C	Spheroplasts frequency % (A–B/A)	Regeneration frequency % C/(A–B)
A. lipoferum[1]	$3.30 \pm 0.4 \times 10^{12}$	$3.20 \pm 0.2 \times 10^{9}$	$1.29 \pm 0.3 \times 10^{12}$	99.0	39.2
A. brasilense[2]	$56.3 \pm 4.3 \times 10^{8}$	$72.0 \pm 5.8 \times 10^{4}$	$44.7 \pm 6.8 \times 10^{7}$	99.0	14.9

[1] Present study.
[2] Sindhu and Dadarwal (1985).

the frequency of the regeneration was observed to 39% (in 2% dextran) as given in the Table I. In the close related nitrogen fixing species of *Azosprillum*, *A. brasilense*, a 99% regeneration was observed, but with a 14.9% regeneration frequency by Sindhu and Dadarwal (1985). This indicates that *A. lipoferum* could be a successful model for transformation studies. The total protein profile (data not shown) of transformants compared on SDS-PAGE showed a 68-kDa band, which is absent in parent *A. lipoferum*. Immunoblot experiment confirmed the physical presence of 68-kDa protein of *cryIC* gene in the transformants and in the positive control pSB607, but absent in the parent *A. lipoferum*. The partial characterization studies of transformants by PAGE, Western analysis and regeneration analysis indicated the successful integration of lepidopteran toxin gene, *cryIC* into nitrogen-fixing *A. lipoferum*.

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Agarwala R. and Tilak K. V. B.R. (1988), Colonization of *Azospirillum* in leaves of *Elusine coracana* and *Setaria itallica*. Zentralbl. Mikrobiol. **143**, 533–537.

Alam M. F., Datta D., Abrigo E., Vasquez A., Senadhira D. and Datta S. K. (1998), Production of transgenic deepwater rice plants expressing *Bacillus thuringiensis cryIA(b)* with enhanced resistance to yellow stem borer. Plant Sci. **135**, 25–30.

Bashan Y., Singh M., and Levanony H. (1989), Contribution of *Azosprillum* to growth of tomato in not through nitrogen-fixation. Can. J. Bot. **67**, 2429–2434.

Döbereiner J. and Pedrosa F. O. (1987), N_2-Fixing Bacteria in Non-leguminous Plants. Brock-Springer series in Contemp. Biosci. Sci. Tech., Wisconsin USA. 1–151.

Fani R., Bazzicalupo M., Coianiz P., and Polsinelli M. (1986), Plasmid transformation of *A. brasilense*. FEMS Microbiol. Lett. **35**, 23–27.

Giles K. L., Hellmich R. L, Iverson C. T., and Lewis L. C. (2000), Effects of transgenic *Bacillus thuringiensis* maize grain on *B. thuringiensis* susceptible *Plodia interpunctella* (Lepidoptera : Pyralidae). J. Ecol. Entomol. **93**, 1011–1016.

Gonzalez-Nunez M., Ortego F. and Castanera P. (2000), Susceptibility of Spanish populations of the corn borers *Sesamina noagrioides* and *Ostrinia nubilalis* to a *Bacillus thuringiensis* endotoxin. J. Ecol. Entomol. **93**, 459–463.

James E. K. and Olivares F. L. (1998), Infection and colonization of sugar cane and other graminaceous plants by endophytic diazotrophs. Crit. Rev. in Pl. Sci. **17**, 77–119.

Mandaokar A. D., Goyal R. K., Shukla A., Bisaria S., Bhalla R., Reddy V. S., Chaurasia A., Sharma R.,P., Altosaar I., and Kumar P. A. (2000), Transgenic tomato plant resistant to fruit borer (*Helicoverpa armigera*). Crop Protection. **19**, 307–312

Mcbridge K. E., Svab Z., Schaaf D. J., Hogan P. S., Stalker D. M., and Maliga P. (1995), Amplification of a chimeric *Bacillus* gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. Biotechnology **13**, 362–365.

Puente M. E. and Bashan Y. (1993), Effect of inoculation with *Azospirillum bransilense* strains on the germination and seedlings growth of the giant columnar cardon cactus (*Pachycereus pringlei*). Symbiosis **15**, 49–60.

Rajendran N. and Venkatesan S. (1993), Synergistic action of plant defense chemicals and the endotoxin of *B. thuringiensis* in relation to *Heliotis armigera* and *Spodoptera litura* (Lepidoptera: Noctuidae). In: Proc. of Chem. Ecol. of Phytophagous Insects (Ananthakrishnan T. N., ed.). Pub: Oxford-IBH, New Delhi, 4, 300–311.

Rajendran N., Sivamani E., and Jayaraman K. (1994), Expression of the insecticidal crystal protein gene from a *Bacillus thuringiensis* in a Gram-negative *Pseudomonas fluorescens* mediated by protoplast fusion. FEMS Microbiol. Lett. **122**, 103–108.

Rovira A. D. (1970), Plant root exudates and their influence upon soil microorganisms. In: Ecology of Soil Borne Plant Pathogens (Baker K. F., and Sydner, W. C., ed.). California, UP. Berkeley. 177–184.

Sambrook J., Fritsch. F. E., and Maniatis T. (1989), Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. NY.

Sano Y. and Kagayama S. (1977), J. Gen. Appl. Microbiol. **23**, 183.

Sindhu S. S. and Dadarwal K. R. (1985), Protoplast isolation and regeneration in *Rhizobium* and *Azospirillum*. Curr. Sci. **23**, 20–24.

Walker D. R., All J. N., McPherson R. M., Boerma H. R., and Parrote W. A. (2000), Field evaluation of soybean engineered with a synthetic *cryIAc* transgene for resitance to corn earworm, soybean looper, velvetbean caterpillar and lesser cornstalk borer. J. Ecol. Entomol. **93**, 613–622.

Weiss. B. (1976), Endonuclease II of *Escherichia coli* is exonuclease III. J. Biol. Chem. **251**, 1896.