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Expression of Azide Resistance is Dependent on Fix Genes in *Rhizobium meliloti*

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A clone of *Rhizobium meliloti* pRK 290.7 known from our previous studies to contain DNA homology in *nif* region was conjugally transferred to *nif* HDK, *nif* DK and *fix* ABC mutants of *R. meliloti*. The transconjugants were examined for restoration of mutational defects on alfalfa plants. It was seen that mutational defect was not restored to wild type. It was also found that transconjugants of *nif* HDK and *nif* DK showed higher level of azide resistance while transconjugant of *fix* ABC remained azide sensitive. Transconjugant of wild type *R. meliloti* 102F34 again showed higher level of azide resistance and bigger nodules in comparison to wild type strain. We conclude that *fix* ABC genes are required for expression of azide resistance and azide resistance in *R. meliloti* is not dependent on the activity of nitrogenase.

Key Words: Azide resistance, Nitrogenase, Fix genes, *Rhizobium meliloti*

Introduction

Symbiotic nitrogen fixation by *Rhizobium meliloti* in alfalfa is one of the most extensively investigated systems for understanding the symbiotic process. The key enzyme of nitrogen fixation, nitrogenase, is a complex enzyme and is composed of three type of polypeptides known as MoFe protein subunit α, MoFe protein subunit β and Fe protein. The enzyme subunits are encoded by *nif*D, *nif*K and *nif*H genes respectively (Kennedy et al. 1976, Roberts et al. 1978). Nitrogenase, besides reducing nitrogen to ammonia, also acts on sodium azide and converts it into nitrogen and ammonia (Hardy & Knight 1967). Sodium azide has been used to develop nitrogenase constitutive mutants in *Azotobacter* (Gordon & Brill 1972) and better nitrogen-fixing strain in *Rhizobium leguminosarum* (Ram et al. 1978). The mechanism of azide resistance in symbiotic fixation of dinitrogen and its relationship with nitrogenase is, however, not fully known. A clone of *R. meliloti* pRK 290.7 identified on the basis of homology to *Agrobacterium* DNA and known to contain *nif* DNA (Kashyap 1989) has been analysed to know its role in symbiotic process. The present finding reveals that this clone possesses genes for azide resistance and that the expression of azide resistance depends upon the *fix* ABC genes of *R. meliloti*.

Material and Methods

Strains and Plasmids

R. meliloti 102F34 is wild type. *R. meliloti nif* mutants N 504, N 324 and *fix*-N 202 carry Tn5 insertion in *nif*H, *nif*D and *Fix*A gene, respectively (Corbin et al. 1983). pRK 290.7 has DNA homology in *nif* region (Kashyap 1989). Plasmid pRK 2073, a derivative of pRK 2013 (Figurski & Helinski 1979) and carrying Tn7 insertion in Kanamycin resistence gene was used as helper plasmid in triparental matings.

Plasmid Transfer and Screening of transconjugants

Bacterial matings were performed by mixing loopfuls of cells of donor, recipient and helper strain on YMB plate. YMB contained 10g mannitol, 1g yeast extract, 0.2g $MgSO_4.7H_2O$, 0.5g K_2HPO_4 and 0.1g Nacl per litre of water. After 48 hr of incubation at 30°C, growth area was scraped, suspended in sterile distilled water and plated on Rhizobium minimal medium (RMin) containing 0.45g Na_2HPO_4, 2.0g $(NH_4)_2$ SO_4, 2.0mg $FeCl_3$, 0.1g $MgSO_4$ $7H_2O$, 0.05g $CaCl_2$ and 10g sucrose per litre of water supplemented with 10 μg/ml tetracycline to screen the transconjugants.

Nodulation and Nitrogen Fixation Test

For nodulation test, alfalfa seeds were soaked in sterile distilled water for 30 min and treated with 0.1% $HgCl_2$ and 60% ethanol for 2 min each and washed thrice with sterile distilled water. Treated seeds were put on filter paper bridge contained in a test tube and submerged halfway in nitrogen free nutrient solution. Nutrient solution consisted of a mixture of MKS and Jenson's solution prepared by mixing 500 ml Jenson's solution and 125 ml MKS solution and making final volume to one litre. (MKS solution contained per litre water: 1.25g $CaSO_4$. $2H_2O$, 0.2g $MgSO_4$. $7H_2O$, 0.2g KH_2PO_4. 0.3g Kcl, 2.86mg H_3BO_3, 1.54mg $MnSO_4$, 0.22mg $ZnSO_4$. $7H_2O$, 0.08mg $CuSO_4$. $5H_2O$, 0.09mg H_2MOO_4, 16.8mg $FeCl_3$ and 2mg EDTA. Jenson's solution contained 1 g $CaHPO_4$, 0.2g K_2HPO_4, 0.2g NaCl, 0.2g $MgSO_4$ and 0.1g $FeCl_3$ per litre of water adjusted to *p*H 7.0. Forty-eight hr-old seedlings were inoculated with bacterial suspension. Plants were grown in growth chamber with light and dark period of 12 hr each and examined after 6 weeks for nodulation and nitrogen fixation.

Test of Azide Resistance

This was done on RMin medium. Cells of a bacterial colony were suspended in a drop of sterile distilled water and spotted on a medium containing different concentrations of azide. Growth of cells after 60 hr of incubation at 30°c was considered as azide resistance.

Results and Discussion

It has been shown previously (Kashyap 1989) that pRK 290.7 has DNA homology in *nif* region of *R. meliloti*.

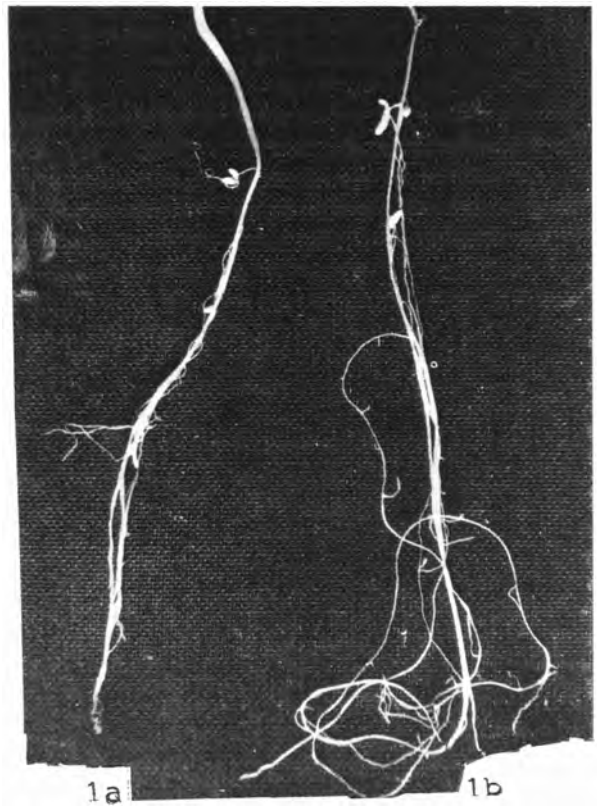

Figure 1 Nodulation on alfalfa plants by 102F34 (1a) and by 102F34 (pRK 290.7) (1b).

Results on nodulation obtained with transconjugants of N504, N324 and N202 in the present study have shown that their mutational defects were not restored to normalcy. Examination of the root nodules, however showed that occasionally intermediate type of nodules were formed on plants with N202, N324 and N504 transconjugants. Transfer of pRK 290.7 to 102F34 yielded larger sized root nodules compared to those formed by 102F34 (figure 1). Acetylene reduction values per plant were two and half times more in nodules of transconjugants than that of the wild type. These results suggested that though pRK 290.7 could not complement the defects in N202, N324 and N504, yet it changed nodule morphology.

Azide resistance of the transconjugants (table 1) revealed that the highest level of azide resistance (4

Table 1 *Test of azide resistance of different strains of* ***Rhizobium meliloti***

Strains	RMin	RMin + tet	RMin + (μg 2	Azide /ml) 3	4
N202	+ + +	—	+ +	VP	—
N324	+ + +	—	+ +	VP	—
N504	+ + +	—	+ +	VP	—
N202 (pRK 290 7)	+ + +	+ + +	+ +	VP	—
N324 (pRK 290.7)	+ + +	+ + +	+ +	+ B	+
N504 (pRK 290.7)	+ + +	+ + +	+ +	+ B	+
102F34 (pRK 290 7)	+ + +	+ + +	+ +	+ B	+
102F34	+ + +	—	+ +	VP	—

+ + +, Normal growth; and + + + B, +, Less than normal growth in the order of decrease, *VP*, Very Poor Growth; — No growth

μm/ml) was shown by N324, N504 and 102F34. The level of azide resistance in mutants N202, N324 and N504 could not be differentiated from the wild type strain 102F34. These results suggested that (i) pRK 290.7 carries genes for azide resistance, (ii) the expression of azide resistance is indepedent of nitrogenase genes because *nif* mutants N324 and N504 were just as sensitive to azide as the wild type strain 102F34, and (iii) the expression of azide resistance is dependent on *fix* ABC genes of *R. meliloti*. Multiplicity of copies per cell of pRK 290.7 (Ditta et al. 1980) may be responsible for increased level of azide resistance in transconjugants.

In response to azide, *fix* genes are expressed in free living cells as evidenced from increased level of resistance of pRK 290.7 carrying N324, N504 and 102F34 cells. The dependence of fix genes on azide resistance and changes in nodulation in 102F34 transconjugants suggests that azide resistance and nodulation are linked processes. Linkage between azide resistance and nodulation has been shown in *R. leguminosarum* (Singh & Kumar 1989), *nif* reiteration has been reported in *R. phaseoli* (Martinez et al. 1985) and *R. japonicum* (Prakash & Atherly 1984). In *R. phaseoli*, *nif*H reiteration has been attributed to specificity of nodulation. Reiteration of *nif*H type in pRK 290.7 may cause both azide resistance and nodulation. It is important to note that *nif*H gene forms a component of nitrogenase and this enzyme in rhizobia is normally expressed in symbiotic cells. However, asymbiotic expression of this enzyme has been reported in stem nodulating rhizobia (Kush et al. 1985). In other rhizobia, asymbiotic fixation of nitrogen has also been reported (Dreyfus et al. 1983). In *R. meliloti*, regulatory gene *nif* A is known to be expressed in free living cells under microaerophilic condition (Ditta et al. 1987). These findings suggest that *Rhizobia* have genetic systems for fixing N_2 both symbiotically and asymbiotically. It is not known whether the same system is responsible for both type of functions. We conclude that azide resistance genes expressed independently of nitrogenase genes and are dependent on *fix* genes. The possibility of azide resistance genes forming a nitrogenase like system in *R. meliloti* is open.

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