CARIBBEAN

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31

Thirty First

Annual Meeting 1995

Barbados

Vol.XXXI

GENETIC STIMULATION OF PURINE AND UREIDE BIOSYNTHESIS IN NODULES OF TRANSGENIC TROPICAL LEGUMES

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ABSTRACT

This project aims to test the hypothesis that an increase in purine biosynthesis and hence ureide biosynthesis in the nodules of transgenic winged bean (*Psophocarpus tetragonolobus*), a tropical legume, will result in improved assimilation of fixed nitrogen, and hence increased overall plant productivity under symbiotic conditions. It necessitates: (i) the development of an efficient *Agrobacterium*-mediated transformation and regeneration protocol for a tropical legume; (ii) the isolation of purine biosynthetic cDNA clones from a mothbean (*Vigna aconitifolia*) nodule library by functional complementation of *Escherichia coli* auxotrophic mutants, and characterization of these clones; and (iii) the production of nodulated transgenic tropical legumes over-expressing glutamine phosphoribosyl amidotransferase (PRAT), the main regulatory enzyme in purine biosynthesis, in the nodules. If the proposed model is successful, this strategy can then be applied to improving the yields of regionally important legumes such as kidney bean (*Phaseolus vulgaris*) and pigeon pea (*Cajanas cajan*).

INTRODUCTION

Rhizobium bacteria form a symbiotic association with leguminous plants whereby the bacteria are accommodated in swellings called nodules on legume roots. The bacteria provide fixed nitrogen to the plant in return for respiratory carbon substrates. As a result of this symbiotic association, leguminous plants are able to thrive in nitrogen-deficient soils.

Symbiotic *Rhizobia* make the single greatest contribution of fixed nitrogen in the nitrogen cycle (Postgate, 1978). This process is particularly important in view of the high energy cost of producing artificial nitrogen fertilizer and the potential environmental problems associated with the widespread use of these fertilizers. The possibility of transferring the capacity for either nitrogen fixation or for forming nitrogen-fixing symbioses to major non-leguminous crops are exciting prospects, and if achieved would undoubtedly rank as a landmark achievement for plant biotechnology. It is not surprising then that this area of plant microbe interaction has been the subject of intense research. Moreover, it offers an opportunity to study the interaction of two genomes, plant (eukaryotic) and bacterial (prokaryotic), in the development of a dynamic organ, the root nodule. Although much progress has been made in characterizing many of the bacterial genes involved, many of the host plant genes

are yet to be characterized, and much work still remains to be done before the possibility of transferring nitrogen fixing capability to non-legumes becomes a reality. However, our present knowledge may permit the improvement of existing symbioses between legume plants and *Rhizobium*. It has been shown that fixed nitrogen is exported from the nodules of tropical legumes as ureides, and from temperate legumes as amides (Schubert, 1986). In tropical legumes, ammonia produced by the *Rhizobia* is first assimilated into glutamine. This is then used to synthesize purines which are subsequently oxidized to ureides (Schubert, 1986; Atkins, 1982).

One of the characteristics of this project is the isolation and characterization of cDNAs encoding enzymes in the purine biosynthetic pathway. In addition to the elucidation of an important metabolic pathway in plants, this information will further illuminate the role of this pathway in the assimilation of fixed nitrogen in nodules. The project also aims to genetically stimulate metabolic flux through the purine and ureide biosynthetic pathways in nodules of a transgenic tropical legume. It is expected that this will enhance the assimilation of fixed nitrogen which may be manifested in increased plant productivity under symbiotic conditions.

Increased purine biosynthesis will be achieved by over-expressing the gene coding for phosphoribosylpyrophosphate amidotransferase (PRAT), the key rate-limiting enzyme in purine biosynthesis (Messenger and Zalkin, 1979; Holmes et al., 1973; Reynolds et al., 1984), in nodules of a transgenic legume. A prerequisite to this experiment is the development of a transformation and regeneration protocol for a tropical legume.

ISOLATION AND CHARACTERIZATION OF cDNA CLONES CODING FOR ENZYMES OF THE PURINE BIOSYNTHETIC PATHWAY

Purine biosynthesis has been best characterized in bacteria and animals, and some studies have also been done in plants. Although the pathway appears to be similar in all organisms studied thus far, there are major differences in the structural organization and regulation of the enzymes (Henikoff, 1987; Chen et al., 1990; Szankasi et al., 1988).

Using functional complementation in *Escherichia coli*, cDNA clones encoding the purine biosynthetic enzymes, PRAT (Delauney et al., 1993; Kim et al., 1995), 5-aminoimidazole ribonucleotide (AIR) carboxylase and 5-aminoimidazole-4-N-succinocarboxamide ribonucleotide (SAICAR) synthetase (Chapman et al., 1994) were previously isolated from mothbean and soybean cDNA libraries. Plant AIR carboxylase and SAICAR synthetase occur in separate polypeptides, while in animals these two enzymes exist as a single bifunctional polypeptide (Chen et al., 1990; Minet and Lacroute, 1990). Differences also occur between plant AIR carboxylase and avian and mammalian AIR carboxylase. There exists some homology between plant AIR carboxylase and the *Schizosaccharomyces pombe* AIR carboxylase in that both possess a similar *purk*-encoded subunit (Szankasi et al., 1988). It is expected that as further plant genes encoding enzymes in this pathway are characterized more differences between plant, animal and bacterial enzymes will be found.

Functional complementation in *E.coli* (Delauney and Verma, 1990) is the major technique which has been used to isolate genes coding for purine biosynthetic enzymes in plants. It involves using particular *E.coli* mutants which are deficient in particular enzymes of the purine biosynthetic pathway by electroporation with a pooled cDNA nodule expression library. An *E.coli* mutant, transformed with the gene encoding the deficient enzyme, will be functionally complemented, i.e. it will now be able to grow on media lacking a source of purines, thereby permitting the isolation of the cDNA clones of interest.

We have recently used this method of functional complementation to isolate four additional purine synthesis cDNAs encoding the enzymes adenylosuccinate lyase, glycinamide ribonucleotide synthase, aminoimidazole ribonucleotide synthetase, and glycinamide ribonucleotide transformylase-N in the purine biosynthetic pathway (unpublished data). These clones will be fully characterized by restriction mapping, DNA sequencing and functional assays where appropriate. This brings the total number of clones encoding for purine biosynthetic enzymes that we have isolated to seven, out of the 10 enzymes in the pathway (see Figure 1). Recently, two other research groups have reported the isolation of *Arabidopsis thaliana* purine synthesis cDNA clones using functional complementation in *E. coli* (Senecoff and Meagher, 1993; Schnorr et al., 1994).

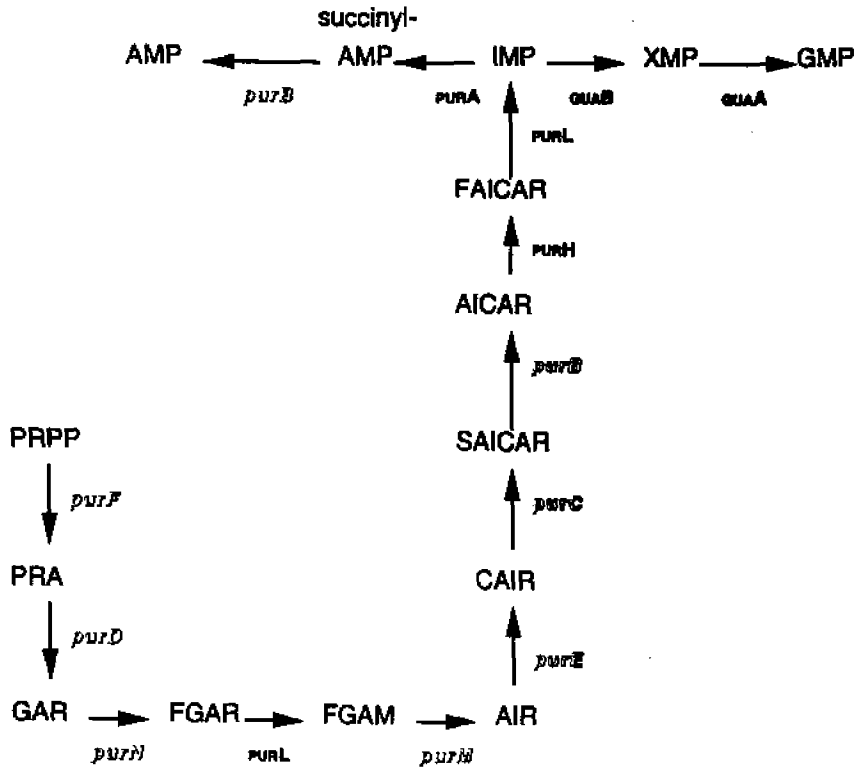

Figure 1 Schematic representation of purine biosynthesis

The individual reactions of the pathway are identified by their gene symbols (*E. coli* nomenclature). PRPP, phosphoribosylpyrophosphate; PRA, phosphoribosylamine; GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; FGAM, formylglycinamidine ribonucleotide; AIR, aminoimadizole ribonucleotide; CAIR, carboxyaminoimidazole ribonucleotide; SAICAR, aminoimidazolesuccinocarboxamide ribonucleotide; AICAR, aminoimidazolecarboxamide ribonucleotide; FAICAR, formamidoimidazolecarboxamide ribonucleotide; PurF, PRPP amidotransferase; purD, GAR synthetase; purN, GAR transformylase N; purL, FGAR amidotransferase; purM, AIR synthetase; purE, AIR carboxylase; purC, SAIR synthetase; purB, adenylosuccinate lyase; purH, AICAR formyltransferase; purA, adenylosuccinate synthetase; guaB, IMP dehydrogenase; and guaA, GMP synthetase. The seven enzymes complemented by *Vigna* cDNAs (purB, purC, purD, purE, purF, purM and purN) are shown.

DEVELOPMENT OF A TRANSFORMATION AND REGENERATION PROTOCOL FOR A TROPICAL LEGUME

In order to produce a transgenic tropical legume over-expressing the PRAT gene, it will be necessary to develop a transformation and regeneration protocol for a tropical legume. This will also facilitate studies on genes which are expressed in nodules of tropical legumes only, such as those involved in ureide biosynthesis. We are currently working with two tropical legumes, mothbean (*Vigna aconitifolia*) and winged bean (*Psophocarpous tetragonolobus*) to develop such a protocol. These legumes were chosen because of published reports indicating that they were amenable to efficient *in vitro* regeneration (Eapen et al., 1986; Gregory et al., 1990).

We have begun experiments on *Agrobacterium* -mediated transformation of mothbean and winged bean. Leaf explants are infected with a disarmed hypervirulent strain of *A. tumafaciens* containing a binary Ti plasmid (pAD582), which confers resistance to the antibiotics kanamycin and hygromycin in plants, and also contains a chloramphenicol acetylase (CAT) reporter gene. After 2 days of co-culture, the explants are placed on selective media containing hygromycin (20 µg/ml) to screen for transformed plant cells, and cefotaxime (500 µg/ml) to kill the *Agrobacterium*. Earlier work (Alleyne and Delauney, 1993) showed that mothbean and winged bean explants were resistant to high levels of kanamycin but were sensitive to hygromycin.

Using *Agrobacterium* strains AGL0 and EHA105, we have been able to obtain putatively transgenic winged bean callus growing on selective media. Further analysis using Southern blotting and CAT assays are necessary to confirm that the callus is genuinely transgenic.

Regeneration of whole plants from untransformed mothbean and winged bean from leaf explants is presently being routinely obtained, with hardened regenerated plants being

transferred to soil (unpublished data). It is therefore expected that the regeneration of transformed plants will soon be achieved.

GENETIC MODIFICATION OF THE PURINE BIOSYNTHETIC PATHWAY

In addition to its central functions in the production of nucleic acids, vitamins, ATP and other co-enzymes, the purine biosynthetic pathway takes on an increased importance in the nodules of tropical legumes since purines are precursors of ureides (Schubert 1986; Atkins, 1991). Thus, there is a marked increase in purine synthesis in nodules of tropical legumes with the onset of nitrogen fixation (Atkins, 1991). Also, the inhibition of ureide production results in the accumulation of purines (Atkins, 1991; Lee et al., 1993).

Evidence from studies in bacteria and animals suggest that PRAT, the first enzyme in the purine biosynthetic pathway, controls the flow of metabolic flux through the pathway by end-product inhibition. Our strategy aims to genetically stimulate the production of the PRAT enzyme in nodules of transgenic legumes. A PRAT cDNA (which we have already isolated) will be linked to a soybean *leg*haemoglobin (Lb) promotor fragment (obtained from DPS Verma, Ohio State University), and introduced into winged bean. The Lb promotor will ensure strong expression of PRAT activity in nodules of transgenic plants (Stougaard et al., 1987; Lee et al., 1993). Transgenic plants will be nodulated and grown in the absence of nitrogen to determine if an increase in purine biosynthesis leads to an increase in the assimilation of fixed nitrogen which may be manifested in improved plant productivity under symbiotic conditions.

It will also be interesting to see what effect an antisense PRAT construct will produce in transgenic nodules. It is expected that there will be inhibition of PRAT activity, and hence reduced purine and ureide biosynthesis in the nodule. This should greatly reduce the assimilation of fixed nitrogen and induce symptoms of nitrogen deficiency, unless tropical legumes are able to activate alternative routes for nitrogen assimilation under these conditions. Thus, the experiment will reveal whether the formation of ureides via purine synthesis is the sole route of fixed nitrogen in tropical legumes, or whether they can resort to alternative pathways of assimilating nitrogen.

SUMMARY AND FUTURE PROSPECTS

This project seeks to address three main areas: (i) the characterization of an important metabolic pathway in plants, the purine biosynthetic pathway by analysis of cDNa clones for the individual enzymes; (ii) the development of a transformation and regeneration protocol for a tropical legume; (iii) improvement of the productivity of tropical legumes under symbiotic conditions by genetic stimulation of the purine biosynthetic pathway.

If the genetic stimulation of purine and ureide biosynthesis in the nodules of transgenic legumes proves to be successful in improving plant productivity under symbiotic conditions, then the same strategy can be applied to the enhancement of plant productivity in other regionally important legumes such as pigeon pea (*Cajanus cajan*) and kidney bean

(*Phaseolus vulgaris*). Moreover, the transformation/regeneration system being developed will allow studies on the regulation of gene expression in tropical legumes. Overall, this research will contribute to the existing body of knowledge on legume-*Rhizobium* symbiosis, thereby bringing us one step closer to being able to transfer nitrogen fixing ability to non-legumes.

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