

# MANIPULATING SUCROSE METABOLISM WITH A SINGLE ENZYME: PYROPHOSPHATE-DEPENDENT PHOSPHOFRUCTOKINASE (PFP)\*

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## Abstract

PFP activity was manipulated in transgenic sugarcane plants to investigate its role in sucrose metabolism. Transgenic plants growing under glasshouse conditions expressed the specific transgenes and had varying levels of PFP activity. Expression of an untranslatable form of the PFP- $\beta$  gene reduced the expression of the endogenous gene by up to 40 and 80% in leaf roll and internodal tissue respectively. Compared to control plants, hexose concentrations increased significantly in the young internodes of these transgenic clones while the sucrose content did not change. In the older internodes hexose levels were slightly higher than that of the control plants while the sucrose content increased significantly. Increases in PFP activity, due to the expression of exogenous PFP genes, was less pronounced in leaf roll tissue of young transgenic plants where the exogenous PFPs contributed up to 20% of the total PFP activity.

**Keywords:** pyrophosphate-dependent phosphofructokinase, sucrose metabolism, carbon flux, sugarcane, transgenic plants

## Introduction

Pyrophosphate-dependent phosphofructokinase (PFP, EC 2.7.1.90) catalyses the reversible conversion of fructose 6-phosphate (F-6-P) and pyrophosphate (PPi) to fructose 1,6-bisphosphate (Fru-1,6-P<sub>2</sub>) and inorganic phosphate (Pi) (Carnal and Black, 1979). The exact physiological role of PFP in plants still needs to be determined but it has been implicated in stress metabolism (Murley *et al.*, 1998; Teramoto *et al.*, 2000), regulation of glycolytic carbon flow (Hajirezaei *et al.*, 1994; Whittaker and Botha, 1999), regulation of sink strength (Edwards and ap Rees, 1986; Black *et al.*, 1995) and PPI metabolism (Black *et al.*, 1987; ap Rees *et al.*, 1988). Down-regulation of PFP activity in transgenic tobacco and potato plants suggests that PFP does not play an essential role in plant metabolism (Hajirezaei *et al.*, 1994; Paul *et al.*, 1995). Although there is a marked reduction in 3-phosphoglycerate (3-PGA) and PEP in these plants, there is little effect on growth and morphology.

In contrast with these indifferent findings in non-sucrose storing plants PFP activity seems to be closely associated with sucrose accumulation in sugarcane, where PFP activity decreases with culm maturity and varies between varieties differing in their sucrose storage capacities. Moreover, PFP activity is inversely correlated with sucrose content across both commercial varieties and a segregating F1 population (Whittaker and Botha, 1999). The only way in which the role of PFP in sugarcane sucrose metabolism can be confirmed, is to manipulate its activity in transgenic plants.

PFP activity can be reduced in transgenic plants by down-regulating the expression of the catalytic subunit (PFP- $\beta$ ) using antisense or co-suppression strategies (reviewed by Iyer *et al.*, 2000). In contrast, over-expressing exogenous, non-plant PFP genes not sensitive to the endogenous regulatory strategies can up-regulate activity.

## Material and Methods

### *Gene isolation and vector construction*

To ensure optimal co-suppression mediated gene silencing in transgenic sugarcane plants the endogenous PFP- $\beta$  gene sequence was isolated from a sugarcane leaf roll cDNA library. The isolated cDNA fragment was cloned as an untranslatable gene into the plant expression vector pUBI 510 which confers high-level, constitutive gene expression in sugarcane cells (Groenewald *et al.*, 2000).

Two exogenous PFP genes were identified for the up-regulation of PFP activity in sugarcane on the basis of their origin, codon usage, amino acid sequence and regulatory characteristics. The *Giardia lamblia* PFP gene (1635bp, 50%GC) encodes a 59.8kDa monomer while a 43.3kDa protein is encoded by the *Propionibacterium freudenreichii* PFP gene (1215bp, 67%GC). The *G. lamblia* PFP gene was obtained from Dr D Dennis (Performance Plants, Queens University, Kingston, Canada), while the *P. freudenreichii* gene was isolated from gDNA using specific primers. The two genes were first cloned into bacterial expression systems to verify their integrity, whereafter they were also cloned into pUBI 510 for sugarcane transformation.

### *Sugarcane transformation*

Standard tissue culture and transformation protocols were used (Snyman *et al.*, 1996). Embryogenic calli were co-transformed with the *npt II* gene and transformed calli were selected on geneticin-containing medium. Geneticin resistant plants were hardened off and grown under glasshouse conditions.

### *Characterisation of transgenic sugarcane plants*

Leaf roll and internodal tissue was collected from glasshouse grown plants, ground in liquid nitrogen and transferred to an appropriate extraction buffer for DNA, RNA, protein and metabolite extractions. Standard protocols, as described by Ausubel *et al.* (1998) and Whittaker and Botha (1997), were used.

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## Results and Discussion

### *Gene isolation, vector construction and sugarcane transformation*

A sugarcane leaf roll cDNA library clone containing an 1170bp insert was isolated and characterised. Sequence analysis revealed that this fragment was more than 80% homologous to the other plant PFP- $\beta$  genes in the international database. Moreover, this insert represented more than 50% of the PFP- $\beta$  coding sequence and included a 260bp 3'-untranslatable sequence. Both *G. lamblia* and *P. freudenreichii* PFP were expressed in a bacterial expression system and characterised. Based on the kinetic parameters obtained these enzymes should function at least at  $\frac{1}{2}V_{\max}$  in transgenic sugarcane plants. Both enzymes were also shown to be completely active in the absence of Fru 2,6-P<sub>2</sub>. Sugarcane calli were transformed with these three genes and several geneticin resistant clones were successfully hardened off.

### *Molecular characterisation of transgenic plants*

Genomic DNA (gDNA) was extracted from putative transgenic plants and subjected to PCR analysis to confirm the presence of the transgenes. Subsequent Southern-blot analyses also confirmed the stable integration of the transgenes.

Expression of the untranslatable PFP- $\beta$  transgene at the transcriptional level and its possible effect on endogenous PFP- $\beta$  transcript levels was investigated using northern-blot analyses. Total RNA from leaf roll and young internodal tissue was transferred to Nylon™ membranes and probed with the 1170bp PFP- $\beta$  cDNA fragment. The results confirmed that the transgene was expressed in all the clones and tissues analysed. Furthermore, the results showed that some of the transgenic tissues had reduced levels of the endogenous PFP- $\beta$  transcript. In comparison to wild type levels, this reduction was especially evident in the internodal tissues where PFP levels were lower than in leaf roll and very young, e.g. internode three, tissue.

Protein levels of the sugarcane PFP- $\beta$  subunit, *G. lamblia* and *P. freudenreichii* PFP were analysed by means of western blot analyses. Total protein of each sample was analysed using SDS-PAGE, transferred to nitro-cellulose membranes and probed with the appropriate antiserum. In all the cases a polypeptide of the expected size cross-reacted with the antibody. The hybridisation signals of PFP- $\beta$  were quantified using spot densitometry, which indicated that the concentration of the protein was reduced in the transgenic plants. Again this was especially evident in the tissues with lower levels of PFP, e.g. internode five. PFP- $\beta$  expression was reduced by up to 40 and 80% in leaf roll and young internodal tissue respectively. This attests to a reduction in PFP activity as there is a reported correlation between the amount of PFP- $\beta$  protein and PFP activity (Whittaker and Botha, 1999).

### *Influence on sucrose accumulation*

Sugars were extracted from internodal tissue of glass house grown control (NCo310) and transgenic plants in which PFP activity has been down-regulated and quantified enzymatically. Hexose concentrations were found to be much higher, up to

eight times, in the youngest transgenic internodes analysed, e.g. internode seven and younger, while the sucrose concentrations did not differ significantly from that of the control plants. In contrast, the older internodes had similar or slightly elevated hexose levels but, compared to NCo310, the sucrose content of some of the transgenic clones increased significantly.

These results suggest that the down-regulation of PFP activity does indeed influence sucrose metabolism in transgenic sugarcane plants. Its influence on other phenotypic traits and the underlying mechanisms are currently being investigated in our laboratories.

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