ELUCIDATION OF A PERIBACTEROID MEMBRANE-BOUND bHLH TRANSCRIPTION FACTOR REQUIRED FOR LEGUME NITROGEN FIXATION

A THESIS SUBMITTED BY

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I Abstract

Many legumes, including soybean, are agriculturally important crop plants. Legumes are unique in their ability to form an endo-symbiosis with soil borne bacteria collectively called rhizobia, which allows the plant to access atmospheric di-nitrogen via the bacteria. The interface between the legume and differentiated, intracellular rhizobia (called bacteroids) is a plant derived membrane called the peribacteroid membrane (PBM). This membrane has a unique complement of proteins, which are required to maintain the bacteroids' environment and allow bi-directional transport of solutes. One such PBM protein from soybean is GmSAT1 (*Glycine max* symbiotic ammonium transporter 1) which was initially characterised as a PBM-localised ammonium transporter based on its ability to complement an ammonium transport-deficient yeast strain 26972c (Kaiser et al., 1998). Subsequent research however, has suggested that GmSAT1 is not directly involved in ammonium transport (Marini et al., 2000).

This project sought to shed some light on the functional role of this intriguing protein. GmSAT1 is unusual in that it has both high homology with known transcription factors of the basic Helix-Loop-Helix (bHLH) family, as well as a predicted C-terminal transmembrane domain. Conservative amino acid substitutions within the bHLH transcription factor domain of GmSAT1 completely abolished the ability of the protein to complement growth of the ammonium transport-deficient yeast 26972c on low ammonium medium. The localisation of GmSAT1 in both soybean and a yeast expression system were examined in depth using immunolocalisation, western blotting of subcellular protein fractions, and GFP fusion proteins. Immunogold

labelling of rhizobia-infected nodule cells verified the localisation of GmSAT1 to the PBM (Kaiser et al., 1998) and additionally the protein was localised in the nucleus. Western blotting demonstrated that GmSAT1 is present as two different size proteins in soybean nodules, with the full length protein present in the insoluble fraction and a truncated protein present in the soluble protein fraction. Biochemical evidence in yeast using a modified two-hybrid reporter system suggests that the GmSAT1 protein, either the full length protein or the N-terminal part, is localised to the nucleus. GmSAT1-GFP fusion protein was localised to small punctate bodies around the yeast cell, adjacent to the plasma membrane and in some instances co-localised with a nuclear stain, also suggesting nuclear localisation.

A soybean genetic transformation protocol was developed to examine the role of GmSAT1 in the symbiosis between soybean and *Bradyrhizobium japonicum* through RNAi gene silencing. Results suggest that GmSAT1 is essential for normal nodule development, with GmSAT1-silenced (*sat1*) nodules being smaller and ineffective in providing the soybean plant with sufficient fixed nitrogen. Rhizobia-infected cells in *sat1* nodules were distinctive in that they retained central vacuoles and did not increase in size and consequently there were far fewer bacteroid located in these cells.

Taken together, our results suggest that GmSAT1 is a membrane-bound transcription factor, located in rhizobia-infected nodule cells of soybean. Upon an as yet undetermined signal, GmSAT1 is proteolytically cleaved from the membrane and imported into the nucleus to activate gene transcription. The functional role of GmSAT1 *in planta* is yet to be determined, however silencing data suggest that it is essential for the maintenance of effective nodules.

II Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

Patrick Charles Loughlin
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IV Abbreviations

bHLH	Basic Helix-Loop-Helix
BLAST	Basic local alignment tool
BSA	Bovine serum albumin
cDNA	Complementary DNA
CaMV	Cauliflower mosaic virus
Cub	C-terminal ubiquitin
ddH_2O	Double distilled H_2O
dsRNA	Double stranded RNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
EMS	Ethylmethane sulfonate
ER	Endoplasmic reticulum
GES	Goldman-Engelmen-Steitz
GFP	Green fluorescent protein
GUS	β-glucoronidase
HIS	Histidine
hpRNA	Hairpin RNA
kB	Kilobase
kDa	Kilodalton
LB	Luria broth (medium)
LZ	Leucine zipper
MA	Methylammonium (chloride)
MeJA	Methyl jasmonic acid
MES	2-[N-Morpholino]ethanesulfonic acid
MW	Molecular weight
NCR	Nitrogen catabolite repression
Nub	N-terminal ubiquitin
OD	Optical density
ORF	Open reading frame

PBM	Peribacteroid membrane
PBS	Peribacteroid space **OR** Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethylsulfonyl fluoride
Pro	Proline
RPM	Revolutions per minute
RNA	Ribonucleic acid
RNAi	RNA interference
rRNA	ribosomal RNA
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
ssDNA	Salmon sperm DNA
TAE	Tris acetate EDTA
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TE	Tris EDTA
TF	Transcription factor
TMD	Transmembrane domain
Tris	Tris(hydroxymethyl)aminomethane
UPR	Unfolded protein response
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucoronic acid
YEM	Yeast extract mannitol (medium)
YNB	Yeast nitrogen base (medium)
YPAD	Yeast extract peptone adenine dextrose (medium)

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