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

A THESIS SUBMITTED BY

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# *3. Subcellular Localisation of GmSAT1*

## 3.1 INTRODUCTION

Transcription factors (TFs) are potent regulators of cellular function, commonly modulating the expression of numerous target genes. As such the regulation of TFs is often tightly regulated. As well as being regulated transcriptionally themselves, TFs are commonly regulated post-translationally, which allows for a rapid response to external and internal stimuli. Many studies, particularly in animals, have examined the multitude of strategies employed by organisms to regulate their TF activity. For example, the active form of a TF is often susceptible to ubiquitin-mediated degradation, allowing for the maintenance of only low levels of active TFs at a given point in time (Desterro et al., 2000). Conversely, other TFs autoregulate their own transcription, essentially creating a positive transcriptional feedback loop (Serfling et al., 2006). Localisation outside of the nucleus is another common way in which organisms regulate TF activity. The mammalian TF NF-κB, which is involved in the immune response, is sequestered in the cytoplasm by the proteolysis-resistant protein IκB. Upon stimulation, IκB is phosphorylated and rapidly degraded, releasing NF-κB, and allowing its import into the nucleus to activate a transcriptional response (Henkel et al., 1993). In the case of the yeast phosphate uptake/assimilation regulator Pho4, the phosphorylation state of the TF itself determines its cytoplasmic/nuclear localisation (Oneill et al., 1996; Komeili and O'Shea, 1999).

### 3.1.1 MEMBRANE-BOUND TRANSCRIPTION FACTORS

Another means of sequestering transcription factors outside of the nucleus is through the anchoring of the protein to an intracellular membrane. The best studied example of this type of post-translational regulation is the mammalian bHLH-leucine zipper (LZ) TF, SREBP (sterol responsive binding element protein), which is anchored to the ER membrane by two transmembrane domains (Espenshade, 2006). Low cellular sterol or ER stress signals the trafficking of SREBP from the ER to the Golgi apparatus where it undergoes two proteolytic cleavage events and is translocated to the nucleus to upregulate genes involved in lipid metabolism (Espenshade, 2006; Colgan et al., 2007). In the fission yeast *Schizosaccharomyces pombe*, an analogous process involving the SREBP-like transcription factor Sre1 occurs in response to hypoxic conditions (Hughes et al., 2005). In mammals, basic leucine zipper (bZIP) TFs involved in the ER stress response have been shown to be cleaved from internal membranes under ER stress and translocate to the nucleus to upregulate genes involved in the unfolded protein response (UPR) (Ye et al., 2000; Murakami et al., 2006; Kondo et al., 2007). A similar ER stress response is also thought to occur in plants, and the *Arabidopsis* bZIP TF AtbZIP60, which has a predicted transmembrane domain, has been shown to upregulate genes involved in the UPR (Iwata and Koizumi, 2005). More recently it has been demonstrated that another *Arabidopsis* bZIP TF, AtbZIP17, is bound to the ER membrane, and is cleaved in response to salt stress (Liu et al., 2007). Additionally a membrane-bound member of the *Arabidopsis* NAC TF family, AtNTM1, which is involved in cell division has also been characterised recently (Kim et al., 2006) and several other NAC TFs are predicted to contain transmembrane domains (Kim et al., 2007). Despite sharing little or no homology with these membrane-bound transcription factors, GmSAT1 does share the

structural similarities of having both a predicted DNA binding motif (the bHLH domain) and a predicted C-terminal transmembrane domain (Figure 3-11). In this chapter several lines of evidence will be presented which suggest that the GmSAT1 protein is anchored to intracellular membranes in both soybean and yeast and that it is released from these membranes and translocated to the nucleus, potentially to activate target gene expression.

## 3.2 RESULTS

### 3.2.1 GmSAT1 IS PRESENT AS TWO DIFFERENT SIZE PROTEINS IN THE SOYBEAN NODULE AND YEAST

In the initial characterisation of GmSAT1, Kaiser et al., (1998) demonstrated by Western blotting that GmSAT1 is present as a ~43 kDa product in the peribacteroid membrane (PBM) fraction of soybean nodules. This finding was re-examined in this study as the high homology of GmSAT1 with basic Helix-Loop-Helix (bHLH) transcription factors and mutagenesis results described in Chapter 2 suggested the possibility of GmSAT1 also being present in a soluble nuclear form. Total soybean nodule proteins were fractionated into soluble and microsomal fractions using centrifugation, separated by SDS-PAGE and Western blotted using anti-GmSAT1 serum. As shown in Figure 3-1A (lane 2), a single band was detected with the GmSAT1 anti-serum in the microsomal fraction, which includes predominantly PBM, corresponding to the previously published expected molecular weight of full length GmSAT1 (~42 kDa) (Kaiser et al 1998). Interestingly, a smaller immunogenic band of approximately 37 kDa was also detected in the soluble nodule fraction (Figure 3-1A, lane 1). This smaller band may potentially be a cross-reactive protein or may be a

truncated version of GmSAT1. To examine this more closely, Western blot analyses of crude protein extracts of yeast, with or without expression of GmSAT1 were completed. Similar to what was observed in soybean, the anti-GmSAT1 serum identified two immunogenic bands in yeast expressing GmSAT1 (Figure 3-1B, lane 2), with the larger band being approximately 39 kDa and the smaller band 35 kDa. The reason for the molecular weight discrepancy (3-4 kDa) between soybean and yeast is unknown but may be due to different posttranslational modification(s) of the protein in soybean. What is notable is that the molecular weight difference between the larger and smaller bands in both yeast and soybean is approximately the same (4-5 kDa) and corresponds to the expected MW of the predicted transmembrane domain of GmSAT1. Western blotting of yeast expressing a mutant GmSAT1 with its predicted transmembrane domain removed (ΔTMD GmSAT1) results in a similar size protein to that of the cleavage product of the full length GmSAT1 (Figure 3-1C, lanes 6 & 7). However this ΔTMD GmSAT1 was unable to complement growth of the yeast strain 26972c on low ammonium medium or induce toxicity when grown on 100 mM methylammonium (data not shown).

Protein from yeast expressing GmSAT1 was also separated into soluble and insoluble fractions and subjected to Western blot analysis using the anti-GmSAT1 serum (Figure 3-1D). The 39 kDa band presumed to be the full length GmSAT1 was present in the membrane fraction (Figure 3-1C, lane 10), as observed by Kaiser et al., (1998), and as would be predicted based on the Western blot analysis of soybean insoluble proteins (Figure 3-1A, lane 2). The level of the 35 kDa product observed in total protein extracts from yeast (Figure 3-1B, lane 2) was reduced in the insoluble yeast fraction, and there appeared to be a concomitant increase in a 29 kDa protein (Figure

3-1C, lane 10), which was only weakly present in the crude yeast protein extract. This 29 kDa band was also present in the soluble yeast protein fraction (Figure 3-1C, lane 2) and may correspond to a further proteolysis product of GmSAT1 derived from the 35 kDa product. The soluble 23 kDa protein (Figure 3-1C, lanes 5 & 7, Figure 3-1D lanes 8 & 9) was a cross-reactive yeast protein sometimes observed in yeast total and soluble protein extracts, in both GmSAT1- and empty vector-transformed yeast.

### 3.2.2 GmSAT1 LOCALISES TO BOTH INTERNAL MEMBRANE SYSTEMS AND NUCLEI OF INFECTED NODULE CELLS

Unpublished immunogold labelling data from Kaiser and colleagues has demonstrated that GmSAT1 is localised to the internal membrane systems of rhizobia-infected nodule cells and tend to form clumps on the PBM (Figure 3-2B & C). As described above, a truncated, soluble form of GmSAT1 was detected in soybean nodules with Western blotting so I sought to localise this soluble form of GmSAT1 using immunogold labelling. A polyclonal GmSAT1-specific antibody was used to localise GmSAT1 protein in 90 nm sections of the infected region of mature soybean nodules. As previously observed, GmSAT1 protein was found to be on the PBM (Figure 3-2A). Further examination across the infected cells also revealed significant immunogold label in the nucleus (Figure 3-3 & 3-4A). The nuclear labelling was not observed in sections probed only with the gold-conjugated anti-rabbit secondary antibody (Figure 4B). The nuclear localisation of GmSAT1 correlates well with the putative role of the bHLH DNA binding domain and the hypothesis that GmSAT1 is a potential transcription factor.

### 3.2.3 GmSAT1 IS LOCALISED TO PUNCTATE, CELL PERIPHERAL VESICLES AND THE NUCLEUS OF YEAST

The subcellular localisation of GmSAT1 in yeast was also determined, using SAT1/GFP fusion proteins. Fusion proteins of GmSAT1 plus N- or C-terminal *mgfp5* (GFP) were constructed as described in the section 3.4.1 and illustrated in Figure 3-5. Similar to un-tagged GmSAT1, N- and C-terminal GFP protein fusions of GmSAT1 were able to complement growth of the ammonium transport-deficient yeast mutant 26972c on 1 mM ammonium plates, and induce MA toxicity (Figure 3-6A), suggesting that fusion of the GFP does not disrupt GmSAT1 function and/or localisation. When expressed alone under the Gal1 promoter, GFP was localised throughout the yeast cytoplasm (Figure 3-7A). When GFP was fused to the C-terminus of GmSAT1, detection of GFP using fluorescence confocal microscopy was problematic. A weak, diffuse GFP signal was detected throughout the yeast cell, however it was difficult to obtain a clear image of its localisation (Figure 3-7B). This was not due to instability of the transcript or protein, as Western blotting demonstrated its expression was similar to untagged GmSAT1 and the N-terminal GFP-tagged GmSAT1 (Figure 3-6B). The N-terminal GFP fusion to GmSAT1 yielded a much stronger GFP signal, detected primarily in punctate vesicles located throughout the cell adjacent the plasma membrane of the cell (Figure 3-7C & D). Additional to these punctate structures, staining live cells with the vital DNA stain hoechst 33342 demonstrated that in some instances the GFP/SAT fusion protein was also co-localised to the yeast nucleus (Figure 3-8).

### 3.2.4 A YEAST TWO-HYBRID ASSAY PROVIDES BIOCHEMICAL EVIDENCE THAT THE N-TERMINAL PART OF THE GmSAT1 PROTEIN IS LOCALISED TO THE NUCLEUS

The yeast two-hybrid system is a robust method in determining specific *in vivo* protein-protein interactions. A yeast two-hybrid system designed to examine interactions between two membrane-bound proteins has been developed (Stagljar et al., 1998). This system makes use of interaction of two proteins bringing into close proximity two halves of a ubiquitin moiety (N-terminal ubiquitin, termed Nub and C-terminal ubiquitin, termed Cub), leading to cleavage of an artificial transcription factor (termed VP16/LexA and consisting of the herpes VP16 transactivating element and bacterial LexA binding domain) from one of the interacting proteins, and activation of reporter genes. Without the protein-protein interaction the VP16/LexA artificial transcription factor remains bound to the membrane associated protein and there is no reporter gene activation (Figure 3-9A). Reporter gene output is measured in the reporter yeast strain DSY-1 by complementation of a histidine auxotrophy and β-galactosidase activity. This technique was originally to be used to examine the possibility that GmSAT1 interacts with the endogenous yeast ammonium transporter Mep3 at the plasma membrane of 26972c. Instead with the mounting evidence that GmSAT1 or a proteolysis product of GmSAT1 is localised to the nucleus of soybean and yeast, I used this technique to provide biochemical evidence of this nuclear localisation. I found that a GmSAT1 construct tagged with an N-terminal VP16/LexA and Cub (pNCW-GmSAT1, see Figure 3-9B) activated both His3 (Figure 3-10A) and lacZ (Figure 3-10C) reporter gene expression in DSY-1, without the presence of the N-terminal half of the ubiquitin moiety and hence independent of ubiquitin-dependent proteolysis. It is probable that only the N-terminal part of GmSAT1 is localised to the nucleus, as when the C-terminus of GmSAT1 was tagged with the artificial

transcription factor (pCMBV4-SAT1; Figure 3-9B) there was no self-activation of reporter gene expression as evidenced by the inability of yeast harbouring this construct to grow on histidine deficient media (Figure 3-10D & E).

### 3.2.5 MUTATION OF A PUTATIVE PROTEOLYTIC CLEAVAGE SITE IN GmSAT1 ABROGATES SELF ACTIVATION OF REPORTER GENE EXPRESSION

Examination of the sequences of mammalian membrane-bound transcription factors identified a known proteolytic recognition sequence 'RXXL' (Murakami et al., 2006) present in GmSAT1, between the transcription factor and transmembrane domains of the protein (Figure 3-11). This site is recognised by the subtilisin-like site-1 serine protease (S1P) (Sakai et al., 1998; Cheng et al., 1999) and although there are no obvious orthologous proteases in *S. cerevisiae* an unrelated subtilisin-like protease may mediate cleavage of GmSAT1 in yeast. Mutation of the RXXL motif has been shown sufficient to prevent proteolytic cleavage of other mammalian membrane-bound transcription factors (Duncan et al., 1997; Ye et al., 2000; Murakami et al., 2006; Kondo et al., 2007). The role of this RXXL putative proteolysis site was examined in GmSAT1 by replacement of the arginine with lysine (R274K) and the replacement of the leucine with alanine, isoleucine or valine (L277A, L277I or L277V), all relatively conservative substitutions. The mutated GmSAT1 cDNAs were fused to the N-terminal artificial transcription factor VP16/LexA and transformed into the yeast reporter strain DSY-1. All substitutions of L277 essentially abolished activation of the HIS3 reporter gene expression (Figure 3-12). Mutation of R274 $\rightarrow$ L caused an intermediate repression of the self activation of the HIS3 reporter gene, with a low level of growth on histidine deficient medium (Figure 3-12). Three GmSAT1 mutants, E177A, L191I and M217L, with amino acid substitutions in their

bHLH domains were also fused to the N-terminal VP16/LexA. None of these mutations affected the self-activation of the HIS3 reporter gene by the VP16/LexA-GmSAT1 fusions (Figure 3-12).

### 3.2.6 MUTATIONS IN THE PUTATIVE PROTEOLYSIS MOTIF AFFECT GmSAT1 FUNCTION IN YEAST

The functional effect of amino acid substitutions in the putative proteolytic cleavage site (RXXL) of GmSAT1 was examined in yeast. A leucine auxotrophic (*leu2)* strain of the ammonium transport yeast mutant 26972c was constructed through the deletion of the LEU2 gene as described in section 3.4.5 to give 26972c Δleu2. Initially 26972c Δleu2 was transformed with wild-type GmSAT1 in the pNCW vector however this construct was unable to complement 26972c Δleu2 when grown on minimal medium with 1 mM ammonium as the sole nitrogen source (data not shown). This was either due to the low expression of GmSAT1 in the pNCW vector, under the cyc1 promoter, or may have been due to the presence of the N-terminal VP16/LexA N-terminal tag. To overcome these problems, 26972c was transformed with the putative proteolysis mutant GmSAT1 in the pYES3 vector, and their ability to complement the yeast strain 26972c on low ammonium and induce MA toxicity were examined. Substitution of R274→K led to an intermediate effect on the self activation of GmSAT1 in the two hybrid studies (section 3.2.5). Interestingly this mutation almost completely abolished MA uptake and associated toxicity, but only weakly affected complementation of the yeast when grown on 1 mM ammonium (Figure 3-13A). Mutations targeting L277 had varying effects on the function of GmSAT1. All retained some toxicity effects associated with methylammonium, with the most

conservative mutation of L277→I showing almost no growth on 100 mM MA, and the less conservative mutations to alanine or valine showing intermediate growth. All three L277 mutants were able to complement 26972c on 1 mM ammonium (weakly in the case of L277V, Figure 3-13A). Western blotting of protein from yeast expressing the RXXL mutant GmSAT1 demonstrated that they were all stable proteins (Figure 3-13B). Additionally, these Western blotting results demonstrated that the RXXL mutations did not affect the presence of the 35 kDa truncated GmSAT1.

### 3.2.7 MODULATION OF THE EXPRESSION LEVELS OF LEU277 GmSAT1 MUTANTS REVEALS FURTHER FUNCTIONAL DIFFERENCES WHEN COMPARED WITH WILD-TYPE GmSAT1

Despite seeing moderate effects in the ability of RXXL mutant GmSAT1 to complement 26972c, and no difference in the proteolysis of GmSAT1, I decided to examine both phenomena more closely. The ability of the L277A and L277V GmSAT1 mutants to complement 26972c on minimal medium supplemented with 1 mM ammonium was examined further through modulation of the expression level of GmSAT1. 26972c yeast expressing the L277A and L277V mutant GmSAT1 were spotted on minimal media supplemented with 1 mM ammonium, with increasing amounts of glucose added to repress Gal1 promoter driven GmSAT1 expression. Although both the L277A and L277V mutant forms of GmSAT1 complement 26972c grown on 1 mM ammonium media when expressed at high levels (2% (w/v) galactose + 0.05% to 0.1% (w/v) glucose, Figure 3-14), differences were observed with increasing glucose concentrations. Wild-type GmSAT1 complemented yeast growth up to 1% (w/v) glucose whereas the L277V and L277A mutants only complemented growth up to 0.5% and 0.1% (w/v) glucose respectively (Figure 3-14). Although not

initially obvious, total levels of GmSAT1 protein were also found to be different when comparing protein extracted from yeast expressing wild-type GmSAT1 versus those expressing the L277A mutant. This difference was only apparent when 0.75% (w/v) glucose was present in the yeast growth medium prior to total protein extraction (Figure 3-14B, lanes 3 & 4).

## 3.3 DISCUSSION

The importance of transcription factors (TFs) in their regulation of cellular activities is reflected in their own activity being tightly regulated. Although TFs are transcriptionally regulated themselves, they are also commonly regulated post-translationally through numerous mechanisms. One post-translational level of regulation is the sequestration of the transcription factor outside of the nucleus. In this study I present several lines of evidence to suggest that the soybean nodule protein GmSAT1, a membrane protein which also shares high homology with characterised basic Helix-Loop-Helix (bHLH) transcription factors is sequestered outside the nucleus by its predicted transmembrane anchor. The transcription factor domain is cleaved from the membrane, allowing the importation of the transcriptionally active form into the nucleus.

### 3.3.1 GmSAT1 IS PROBABLY CLEAVED AT THE SAME SITE IN BOTH YEAST AND SOYBEAN

GmSAT1 is a soybean protein expressed predominantly in nodule tissue, with some expression also detected in infected roots after the nodules have been detached (Kaiser et al., 1998). Probing of Western blots of crude soluble/insoluble nodule

protein extracts revealed the presence of two immuno-reactive bands, a larger 42 kDa band in the insoluble fraction and a smaller band, approximately 37 kDa in the soluble fraction. Previously published Western blot results failed to readily detect the smaller immuno-reactive band in the soluble nodule fraction (Kaiser et al., 1998, Figure 3-4B). However this band was detected in nodule soluble fractions with subsequent Western blot analysis (B. Kaiser, unpublished). It is possible that the smaller 37 kDa band in the soluble fraction corresponds to a cross reactive related protein, possibly another bHLH transcription factor, however the presence of two similar immuno-reactive proteins in yeast expressing GmSAT1, but not in empty vector transformed yeast, suggests both proteins are GmSAT1 products. It is unclear as to why this smaller GmSAT1 protein which is soluble in the soybean nodule appears to be at least in part insoluble in yeast (Figure 3-1D). One possible explanation is the different separation techniques used between soybean and yeast protein extraction. The identity of the ~29 kDa product observed most prominently in both the soluble and insoluble yeast fractions (Figure 3-1D, lanes 9 and 10) and ΔTMD GmSAT1 total protein extracts (Figure 3-1C, lane 7) is most likely a further proteolysis product of the truncated GmSAT1 protein. The different techniques used to extract total versus soluble and insoluble protein may explain the increased proteolysis observed in Figure 3-1D, whereas the most probable cause of the increased presence of this 29 kDa band in ΔTMD GmSAT1 total protein extract is an instability in the mutant GmSAT1 protein.

### 3.3.2 THE DUAL LOCALISATION OF GmSAT1 WHEN EXPRESSED IN YEAST

The subcellular localisation of GmSAT1 in yeast was also tested using GFP and artificial transcription factor fusions. GFP was fused to the N- and C-termini of GmSAT1 and expressed in the yeast ammonium transport mutant 26972c. The N-terminal GFP fusion of GmSAT1 was localised to very distinct, punctate vesicles around the periphery of the yeast cell and in some instances, the fusion protein was also localised in the nucleus of the yeast cell. The identity of the cell-peripheral punctate bodies is unknown however they do resemble yeast eisosomes which are thought to be involved in the endocytosis of plasma membrane proteins (Walther et al., 2006). Unfortunately the C-terminal GmSAT1-GFP fusion protein gave very low GFP signal so it could not be determined if this fusion protein was also localised to these punctate bodies and/or the yeast nucleus. Possibly the environment that the C-terminal GFP fusion protein was located was, for example, highly acidic which is not conducive for its correct folding and hence there was little GFP signal (Gnanasambandam and Birch, 2004). The fact that the C-terminally tagged GmSAT1/artificial transcription factor construct did not activate reporter gene expression whereas the N-terminally tagged GmSAT1/artificial transcription factor did (Figure 3-10) suggests that only the N-terminal portion of the protein is translocated into the nucleus.

### 3.3.3 CLEAVAGE OF GmSAT1 FROM SOYBEAN AND YEAST MEMBRANES?

The sequestration of transcription factors on internal membrane systems and subsequent regulated proteolytic release is a well documented phenomenon, observed in organisms as diverse as bacteria, (Alba and Gross, 2004), animals (Murakami et al., 2006) and more recently plants (Kim et al., 2007; Liu et al., 2007). Such transcription factors are commonly (but not always) involved in responses to cellular stress often specific to their membrane localisation. Assuming a similar proteolytic event is occurring in GmSAT1, the signal leading to the cleavage event and the identity of the putative protease(s) involved in this cleavage are not known. In this study, the possible role of a putative proteolysis recognition motif (RXXL) situated adjacent the predicted transmembrane domain of GmSAT1 was examined. This site is recognised by the site-1 protease (S1P) in the mammalian SREBP, ATF6, OASIS and BBF2H7 membrane bound transcription factors (Duncan et al., 1997; Ye et al., 2000; Murakami et al., 2006; Kondo et al., 2007). In GmSAT1, mutations of this RXXL motif were introduced and the nuclear localisation of the resulting constructs was determined using a modified yeast two-hybrid technique. Results from these experiments indicated that the artificial transcription factor was no longer being imported into the nucleus when the leucine in the RXXL motif was mutated, and a decreased level of reporter gene activation was detected when R274 was replaced with lysine. Interestingly, although arginine is present at this position in all transcription factors known to be processed by the S1P, mutation of this arginine to lysine in the sterol-responsive binding element protein-2 (SREBP-2) did not affect proteolysis (Hua et al., 1996). Initially these results suggested that GmSAT1 was being cleaved at the RXXL site, and that the described amino acid substitutions were

preventing this cleavage event. However Western blotting of protein from yeast expressing GmSAT1 with mutations in the RXXL revealed no change in the 35 kDa putative proteolysis product (Figure 3-13B). Additionally these mutant forms of GmSAT1 were able to complement 26972c at least to some extent (Figure 3-13A), suggesting that the intact RXXL motif is not required for the formation of the truncated GmSAT1. However in the case of the split ubiquitin experiment, the mutant GmSAT1 were expressed under a low expression Cyc1 promoter, whereas with the complementation and Western blotting experiments, GmSAT1 was overexpressed under the Gal1 promoter, which may in part explain the incongruous effects these mutations caused in GmSAT1. Western blotting of the mutant GmSAT1 expressed under the Cyc1 promoter may have answered whether this was the case, however it was not possible to detect GmSAT1 protein by Western blotting when expressed under the Cyc1 promoter (data not shown). It is important to note that the RXXL motif is present in the luminal domain of other transcription factors cleaved by the S1P (Kondo et al., 2007), therefore if GmSAT1 was cleaved at its RXXL site (Figure 3-11) its transcription factor domain would have to face the ER/Golgi lumen, which is the opposite to the other transcription factors described in Figure 3-11 and would therefore require alternative mechanisms to be translocated to the nucleus.

Irrespective of the actual site of proteolysis, Western blotting suggests that a cleavage event is occurring in the GmSAT1 protein and the size of the truncated GmSAT1 corresponds to this cleavage occurring at the N-terminal end of the predicted transmembrane domain. In yeast, fusion of both an artificial transcription factor and GFP to the N-terminus of GmSAT1 demonstrate that at least some of the GmSAT1 protein is nuclear localised. Western blotting and immunogold labelling has

previously localised GmSAT1 to the peribacteroid membrane (PBM) and other endomembranes of soybean infected cells (Kaiser etal., 1998: Kaiser et al., unpub.). In this study as well as the previously reported PBM localisation of GmSAT1, immunogold labelling using GmSAT1 antiserum also demonstrated the presence of immunoreactive protein in the nucleus of bacteroid-infected soybean nodule cells. This nuclear labelling most likely corresponds to the smaller immuno-reactive protein detected with Western blotting of total soluble nodule protein, whilst the PBM associated immunogold labelling probably corresponds to the larger band detected in the insoluble fraction. I would hypothesise that GmSAT1 is a PBM-associated protein which under yet to be defined circumstances is released from the PBM or alternatively en-route to the PBM through the ER/Golgi secretory pathway, probably through a regulated proteolytic cleavage event and is imported into the nucleus and involved in the transcriptional regulation of nodule-associated genes.

## 3.4 METHODS

### 3.4.1 VECTOR CONSTRUCTION

Standard DNA manipulations were carried out essentially as described by Sambrook and Russell (2001). *mGFP5* was PCR amplified from the pCAMBIA 1302, minus the 6xHIS tag, with primers with ~ 30bp 5' overhangs specific to pYES3 and GmSAT1 sequences as outlined in Table 3-1. The following primers were used for a) N-terminal GFP-SAT1 - Gal1GFPFW/SATstartGFPRV b) C-terminal SAT1-GFP – SATendGFPFW/CYCtermGFPRV and c) GFP alone – Gal1GFPFW/ CYCtermGFPRV. pYES3-GmSAT1 vector was digested within the region to be

recombined with suitable restriction endonucleases (*Sal* I for the N-terminal GFP-SAT1 construct, *Sph* I and *Not* I for the C-terminal SAT1-GFP construct and GFP alone construct). Both the PCR amplified GFP and digested pYES3-GmSAT1 were used to co-transform the yeast strain 26972c to allow for the recombination of the digested vector with the homologous regions of the GFP PCR product. Selection of correct pYES3-GmSAT1/GFP recombinant plasmids was initially by colony PCR. Plasmid was extracted from putative positive yeast colonies and used to transform DH5α *E. coli*. Plasmid DNA was purified and its identity verified by restriction enzyme analysis.

GmSAT1 and ScMEP3 were cloned into the *Pst* I / *Sac* I sites of pNCW, in frame with the N-terminal VP16/LexA-Cub construct using primers described in Table 3-1. GmSAT1 was PCR amplified from pYES3-GmSAT1 with primers described in Table3-1 (pCMBV4SATFW/RV), which have 40bp 5' overhangs homologous to upstream of the VP16/LexA-Cub regions of the pCMBV4 vector. The yeast reporter strain DSY-1 (MATa his3Δ200 trp1-901 leu2-3, 112 ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ GAL4, Dualsystems$^{TM}$) was co-transformed with *SfiI*-digested pCMBV4 and the GmSAT1/pCMBV4 PCR product described above to allow recombination of the digested vector and homologous regions of the PCR product. Positive recombinants/transformants were initially selected by colony PCR and the construct isolated, used to transform DH5α *E. coli* and verified by restriction enzyme digests.

ΔTMD GmSAT1 was constructed by PCR amplification of the *Gmsat1* cDNA in the pYES3 vector with primers (SATdeltaTMDFW/RV; Table 3-1) designed to loop out

159 nucleotides of the coding region of GmSAT1, corresponding to the amino acids S294-V347, which include the predicted transmembrane domain (amino acids S306-E327).

### 3.4.2 PCR AMPLIFICATION OF 'RXXL' MUTANT *GmSAT1*

Mutagenic PCRs, and selection of resulting mutants were performed as described in sections 2.4.1 and 2.4.2, except that for mutagenic PCRs performed using pNCW-GmSAT1 as template, the extension time was increased from 16 min to 18 min. Primers used for mutagenesis and restriction enzymes used for mutant selection are described in Table 3-2. Mutant cDNAs were sequenced to verify the mutation and that no spurious PCR-induced mutations had occurred.

### 3.4.3 YEAST TECHNIQUES

Yeast were transformed as described in section 2.4.3. Yeast growth studies were performed as described in section 2.4.4. Total protein extraction from yeast, SDS-PAGE and Western blotting were performed as described in sections 2.4.6-2.4.8. For total yeast protein described in Figure 3-14B, yeast were grown to mid-log phase in minimal medium supplemented with 0.1% (w/v) proline, 2% (w/v) galactose and 0.1% or 0.75% (w/v) glucose as indicated.

### 3.4.4 LACZ ACTIVITY DETERMINATION IN YEAST

To assay lacZ activity, DSY-1 yeast (MATa his3Δ200 trp1-901 leu2-3, 112 ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ GAL4, Dualsystems$^{TM}$) with either

empty pNCW or pNCW-GmSAT1 were grown for 48 hours on solid leucine deficient YNB media (w/o amino acids, Difco), to select for the pNCW vector, plus 2% (w/v) glucose, supplemented with tryptophan (40 µg/ml), adenine hemisulfate (40 µg/ml) and histidine (20 µg/ml). Whatmann filter paper (3MM) was placed gently on the yeast colonies and left for 10 min to allow the yeast to stick to the paper. The paper was then immersed in liquid $N_2$ to lyse the yeast cells before being placed colonies facing up, in a new petri dish and allowed to thaw. The paper was overlayed with a warm (50°C) mix of 0.5% (w/v) agarose in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, 1.47 mM $KH_2PO_4$, pH 7.4) containing 0.1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Sigma). The paper was incubated at room temperature for approximately 1 hour to allow development of the blue colour.

### 3.4.5 DELETION OF LEU2 FROM THE 26972C GENOME

Deletion of the LEU2 locus of 26972c was through homologous recombination of a kanMX4 PCR product amplified from pFA6a-KanMX4 (Wach et al., 1994) with primers with Leu2 specific 5' overhangs as described in Table 3-1 (Leu2KanmxFW/RV). 26972c were transformed with the PCR product as described in section 4.4.3 except after the 42°C heat shock, cells were spun down, and resuspended in 1mL YPAD and gently shaken at 30°C for 2-4 hrs before plating on YPAD supplemented with 200 µg/ml G418 (geneticin, Sigma). Colonies which appeared 2-3 days later were restreaked on YPAD/G418 medium to verify geneticin resistance and deletion of the leu2 was determined by PCR using primers Leu2upstreamFW and Leu2internalRV (Table 3-1) and inability of the 26972c strain to grow on leucine deficient medium (data not shown).

### 3.4.6 CONFOCAL FLUORESCENCE MICROSCOPY

26972c and Σ1278b yeast harbouring the pYES3-GFP, pYES3-GFP/SAT and pYES3-SAT/GFP constructs were grown in minimal medium plus 2% (w/v) galactose, supplemented with 1 mM $NH_4Cl$, 35 mM $NH_4Cl$, 0.1% (w/v) proline or 0.1% (w/v) glutamine as nitrogen sources. As there were no observable differences in GFP intensity or localisation between the two yeast strains or nitrogen conditions (data not shown), 26972c yeast harbouring the GFP constructs were routinely used for fluorescence microscopy. The yeast were grown to mid-log phase in Grenson's minimal medium (Grenson, 1966) supplemented with 2% (w/v) galactose and 0.1% (w/v) proline and either viewed directly using a Leica spectral confocal microscope or stained with the vital DNA stain hoechst 33342 at a final concentration of 500 μM, and viewed using a Biorad MRC-1000UV microscope.

### 3.4.7 EXTRACTION AND SEPARATION OF SOLUBLE AND INSOLUBLE PROTEIN YEAST FRACTIONS

26972c yeast harbouring pYES3 or the pYES3-GmSAT1 vectors were grown to mid-log phase in Grenson's minimal media (Grenson, 1966) supplemented with 0.1% (w/v) proline and 2% (w/v) galactose at 28°C with shaking. Total soluble and insoluble protein fractions were prepared from 10 mL of yeast culture. Cells were washed once in extraction buffer (10 mM Tris-HCl, pH7.5, 1 mM EDTA, 1 mM PMSF, plus Complete, mini, EDTA-free protease inhibitor cocktail (Roche) according to the manufacturer's instructions) and resuspended in 200 μl of extraction buffer. To this, approximately 200 mg of 0.45 μm glass beads were added and the mixture

vortexed vigorously for 15 min at 4°C. Glass beads and larger pieces of cell debris were removed by centrifugation at 700 x *g* for 10min at 4°C and the supernatant collected. This supernatant was centrifuged at 125,000 x *g* at 4°C for 2 hr to separate soluble (supernatant) and insoluble (pellet) protein fractions. Soluble protein (supernatant) was pipetted into a new tube and the insoluble protein (pellet) was resuspended in 5% (w/v) SDS and also transferred to a fresh tube. An equal volume of SDS running buffer (section 4.4.6) was added to each and samples subjected to SDS/PAGE and Western blotting as described in sections 4.4.7-4.4.8.

### 3.4.8 EXTRACTION AND SEPARATION OF SOLUBLE AND INSOLUBLE PROTEIN FRACTIONS FROM SOYBEAN NODULES

Soybean (*Glycine max* L. cv. Boyer) were grown in sand under glasshouse conditions. Plants were fertilised twice weekly with a modified Herridge's nutrient solution (minus N; Herridge 500 µM $MgSO_4$, 500 µM $KH_2PO_4$, 500 µM $K_2SO_4$, 50 µM KCl, 20 µM Fe-EDTA, 250 µM $CaSO_4$, 25 µM $H_3BO_3$, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.5 µM $NaMoO_4$) and inoculated with *B. japonicum* USDA110 to induce nodulation. Mature soybean nodules were harvested and ground in a chilled mortar in ice-cold extraction buffer (25 mM MES-KOH pH 7.0, 350 mM mannitol, 3 mM $MgSO_4$, 1 mM PMSF plus Complete, mini, EDTA-free protease inhibitor cocktail (Roche) according to the manufacturer's instructions). The nodule homogenate was filtered through 4 layers of extraction buffer-soaked miracloth (Calbiochem) and centrifuged at 20,000 x *g* for 20 min at 4°C. The supernatant was collected and centrifuged at 125,000 x *g* for 1 hour at 4°C to separate the soluble and total membrane protein fractions. The total membrane fraction (pellet) was resuspended in

5% (w/v) SDS and the soluble fraction (supernatant) was concentrated by precipitation using 6% (w/v) TCA and resuspended in 5% (w/v) SDS. An equal volume of SDS running buffer (section 4.4.6) was added to each and samples subjected to SDS/PAGE and Western blotting as described in sections 2.4.7-2.4.8.

### 3.4.9 IMMUNOGOLD LABELLING OF SOYBEAN NODULE SECTIONS

90 nm sections of soybean nodules were prepared as described in section 4.4.6, and placed on nickel grids. Nickel grids were placed specimen side down on drops of 50 mM glycine for 2 x 10 min. The grids were blotted before being placed on 1% (w/v) BSA in PBS for 15 min. Grids were blotted again and incubated overnight at 4°C on 20 µl primary antibody, diluted 1:100 with 1% (w/v) BSA in PBS. Grids were then rinsed 6 x 5 min on 1% (w/v) BSA in PBS, blotted on filter paper and incubated for 1 hour at room temperature in a goat anti-rabbit 10 nm gold conjugated secondary antibody (Aurion), at 1:100 dilution in 1% (w/v) BSA in PBS. Grids were rinsed 6 x 5 min in PBS, washed briefly in 4 small beakers of dd$H_2O$ and dried using filter paper. Grids were counter stained using 3% (w/v) uranyl acetate in 70% ethanol for 3 min, and examined using a Phillips CM100 electron microscope.

| NAME | SEQUENCE (5' - 3') |
|---|---|
| SATendGFPFW | AAGGATCTTGTGAGAAGTTTACGCTCAGCTTTTTCATATTTCGTG ATGGTAGATCTGACTAGTAAAGG |
| Gal1GFPFW | ATCGGACTACTAGCAGCTGTAATACGACTCACTATAGGGAATATT ATGGTAGATCTGACTAGTAAAGG |
| SATstartGFPRV | AGGTAGCCCTCTGATTGATGAAATCTCCATATGAGAACTCCTCAT AGCTTTGTATAGTTCATCCATGCC |
| CYCtermGFPRV | AGGGCGTGAATGTAAGCGTGACATAACTAATTACATGATGCGGCC TCAGCTAGCTTTGTATAGTTCATC |
| | |
| GmSAT1_PstI_FW | GGCCCTGCAGCATGAGGAGTTCTCATATGGAGAT |
| GmSAT1_SacII_RV | GGCCCCGCGGTCACACGAAATATGAAAAAGCTGA |
| | |
| ScMEP3_PstI_FW | GGCCCTGCAGCATGGCTCGGGGTGACGGACATCTATGG |
| ScMEP3_SacII_RV | GGCCCCGCGGTCATGCTTCTTTTGCGTGATGCAATTTAGG |
| | |
| SATdeltaTMD_FW | GTCATAGAGAAAACAATCTGAAGAGTTATGCATTTTTCG |
| SATdeltaTMD_RV | CGAAAAATGCATAACTCTTCAGATTGTTTTCTCTATGAC |
| | |
| Leu2KanmxFW | CCAATAGGTGGTTAGCAATCGTCTTACTTTCTAACTTTTCCGTAC GCTGCAGGTCGAC |
| Leu2KanmxRV | TTCCATTTTGTAATTTCGTGTCGTTTCTATTATGAATTTCATCGAT GAATTCGAGCTCG |
| | |
| Leu2upstreamFW | CGCCTGACGCATATACCTTT |
| Leu2internalRV | CGAACTTGACATTGGAACGA |
| | |
| pCMBV4SATFW | TACTTCTATAGACACGCAAACACAAATACACACACTAATCTAGAC ATGAGGAGTTCTCATATGGAG |
| pCMBV4SATRV | CATGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGAT CACGAAATATGAAAAAGCTGA |

**Table 3-1. Primers used in Chapter 3.**

| NAME | SEQUENCE (5' - 3') | Restriction site change |
|---|---|---|
| | | |
| R274K_FW | CAAGATTTTGGGAAAAAAATGTACTCATAAGAATAC | RsaI introduced |
| R274K_RV | GTATTCTTATGAGTACATTTTTTTCCCAAAATCTTG | |
| | | |
| L277A_FW | GGAAAGAAATGTGGCCATAAGAATACATTG | HaeIII introduced |
| L277A_RV | CAATGTATTCTTATGGCCACATTTCTTTCC | |
| | | |
| L277I_FW | GGAAAGAAATGTGATCATAAGAATACATTG | BclI introduced |
| L277I_RV | CAATGTATTCTTATGATCACATTTCTTTCC | |
| | | |
| L277V_FW | AAGAAATGTCGTGATCAGAATACATTGTGA | DpnI introduced |
| L277V_RV | TCACAATGTATTCTGATCACGACATTTCTT | |

**Table 3-2. Primers used to introduce point mutations into the 'RXXL' putative proteolytic cleavage site of GmSAT1.** Amino acids substituted are indicated in the first column. Red letters in the primer sequence correspond to mutated nucleotides, both to introduce the amino acid substitutions and silent mutations as required to delete/introduce restriction enzyme sites designated in column 3. These restriction enzyme sites were used to distinguish mutant GmSAT1 cDNAs from wild type GmSAT1.

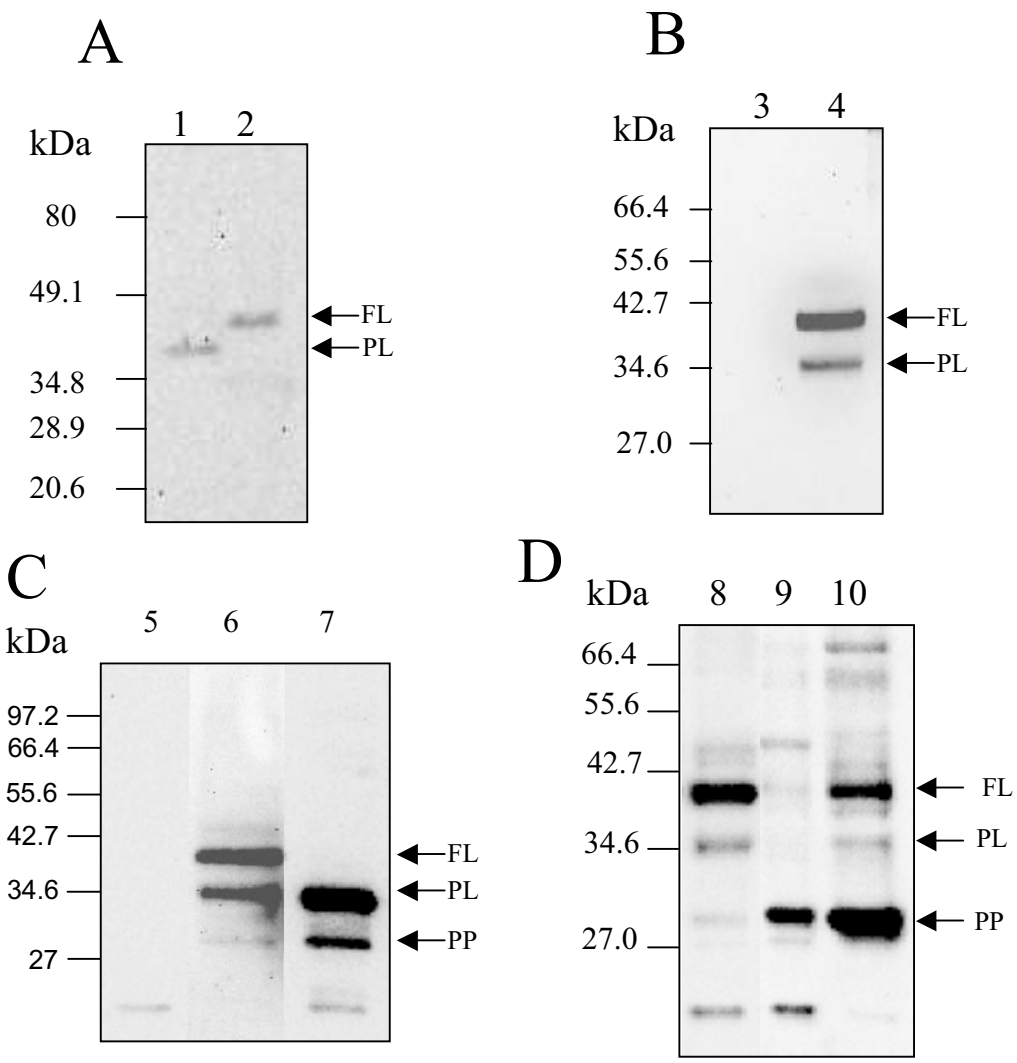
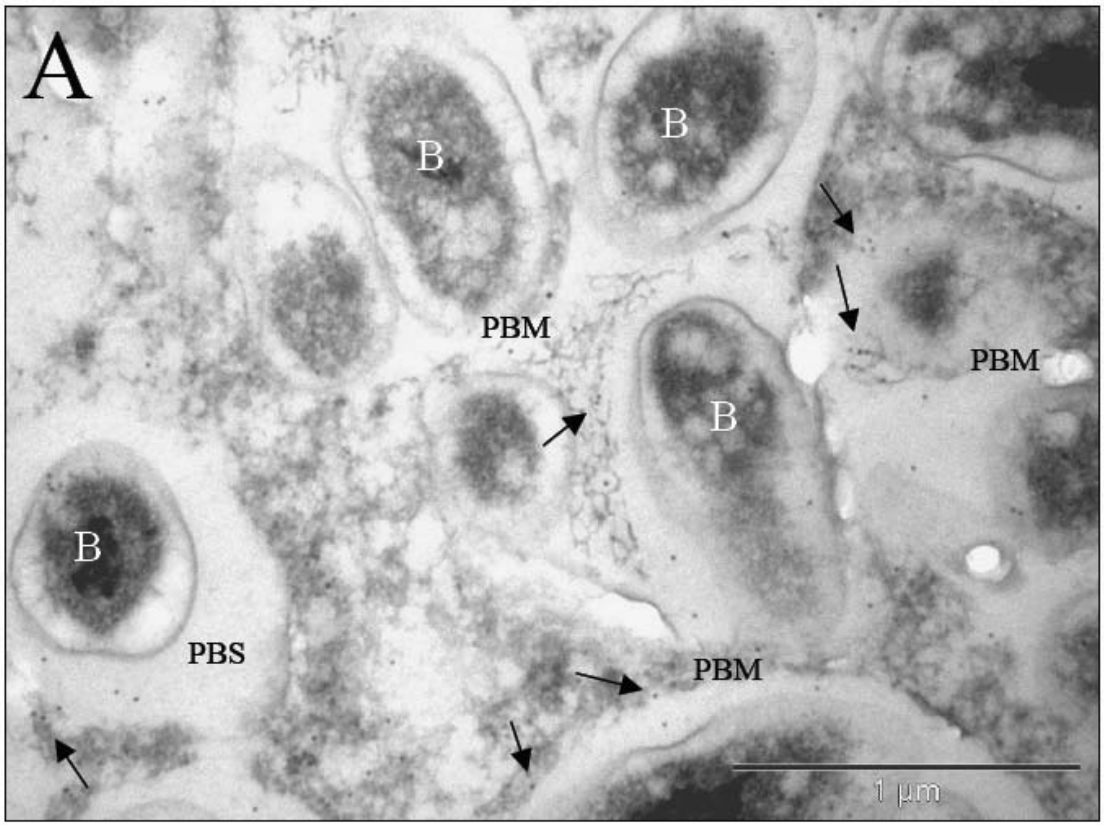



**Figure 3-1. Anti-GmSAT1 serum recognises at least two different size epitopes in both soybean nodules and yeast expressing GmSAT1.** SDS-PAGE and western blotting was performed as described in section 4.4.7 & 4.4.8. **(A)** Total nodule protein was extracted and fractionated into soluble (lane 1) and insoluble (lane 2) fractions as described in section 3.4.8. **(B)** Western blot of total protein from yeast with (lane 4) or without (lane 3) GmSAT1 expression probed with anti-GmSAT1 serum**. C)** Western blot analysis of full length GmSAT1 (lane 6) and the mutagenised GmSAT1 devoid of the C-terminal membrane spanning region (lane 7) expressed in yeast and an empty vector control (lane 5). **D)** Western blotting of total **(**lane 8**),** soluble **(**lane 9**),** and insoluble **(**lane 10**)** protein extracts from yeast expressing GmSAT1 prepared as described in section 3.4.7. Note: FL - full length GmSAT1, PL - GmSAT1 proteolysis product, PP - secondary GmSAT1 proteolysis product.




NOTE: Figures B and C are included on page 3-26 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 3-2. Immunogold-localisation of GmSAT1 to the peribacteroid membrane of infected nodule cells.** Soybean nodule sections were prepared as described in section 2.4.6, probed with anti-GmSAT1 serum (1:100 dilution) followed by gold conjugated anti-rabbit IgG (1:10 dilution) as described in section 3.4.9. Immunogold labelling was predominantly restricted to the peribacteroid membranes (arrows) **(A, B** and **C).** Some GmSAT1 protein was also detected in trafficking vesicles **(B**, triangle**)** and vesicles budding from the ER **(C,** triangle**)** Bbacteroid, PBM- peribacteroid membrane, PBS- peribacteroid space, ERendoplasmic reticulum (figures B and C from Kaiser and Federova (unpublished results).

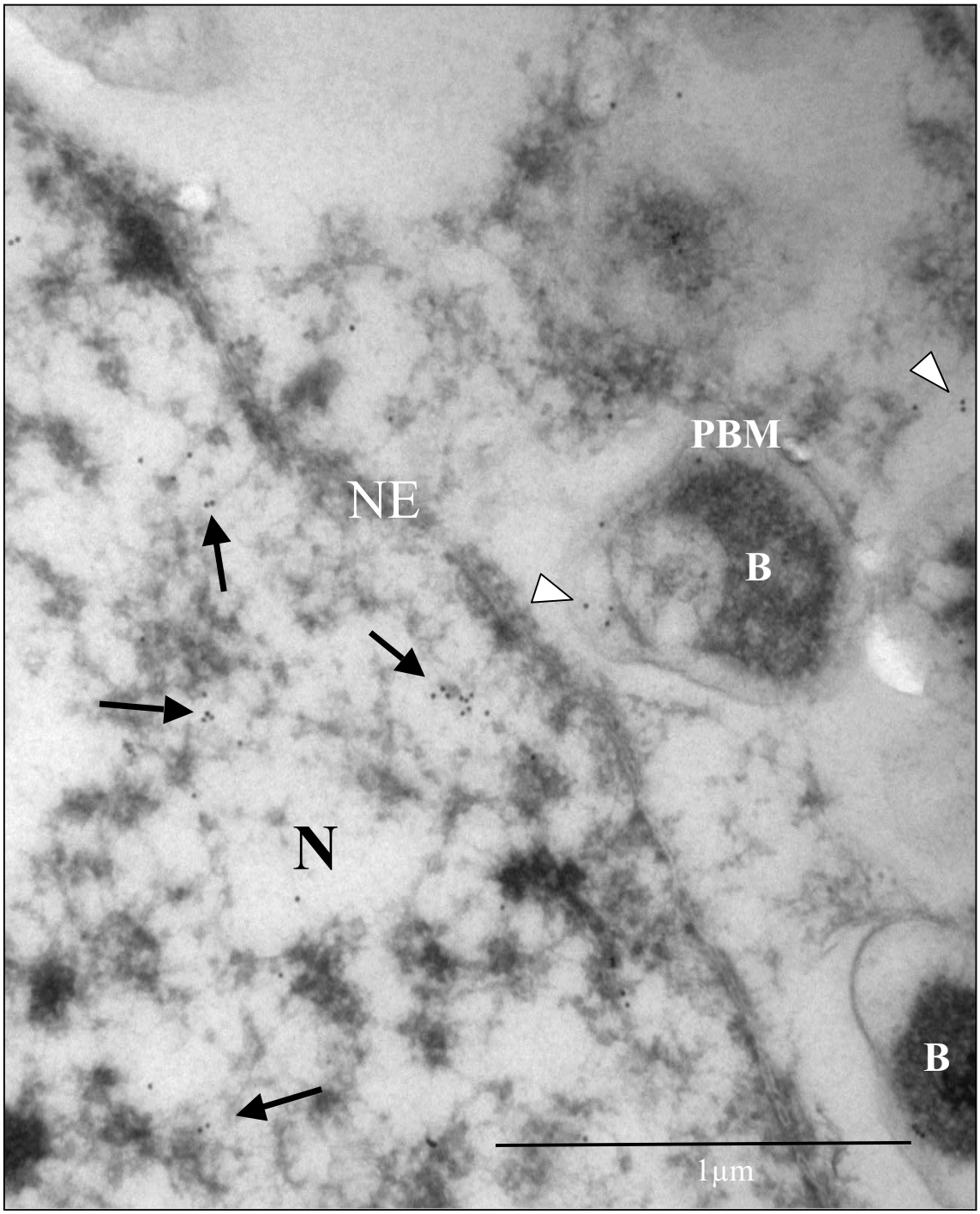



**Figure 3-3. Immunogold-localisation of GmSAT1 to the nucleus and PBM of infected nodule cells.** Soybean nodule sections were prepared as described 2.4.6, The section was probed with anti-GmSAT1 serum (1:100 dilution) followed by gold conjugated anti-rabbit IgG (1:10 dilution) as described in section 3.4.9. Clumps of immunogold labelling were localised within the nucleus (arrows) and peribacteroid membrane (triangle). N, nucleus NE, nuclear envelope B, bacteroid PBM, peribacteroid membrane

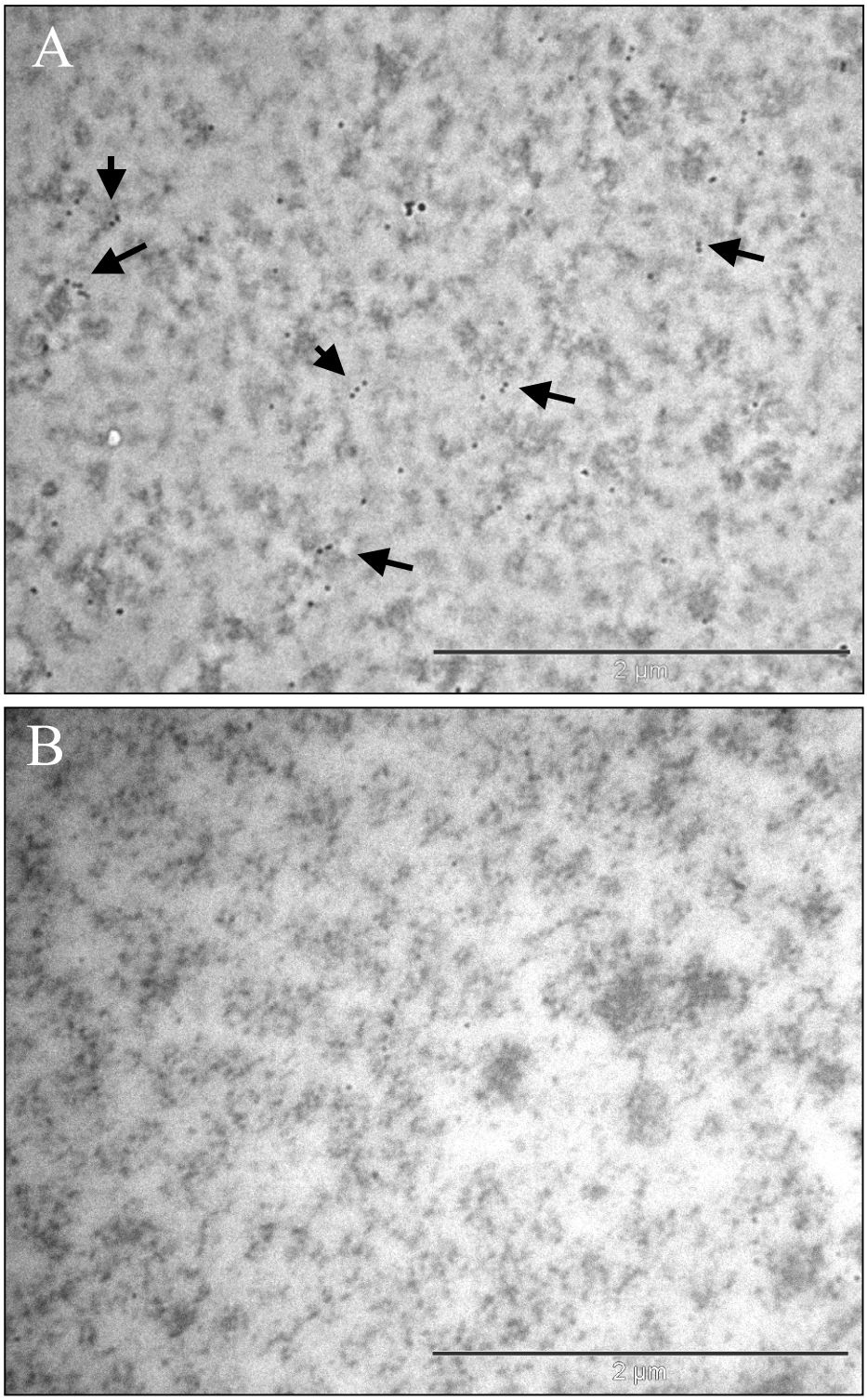



**Figure 3-4. GmSAT1 antiserum is necessary to localise immunogold-conjugated IgG to the nucleus of infected nodule cells.** Soybean nodule sections were prepared as described in section 2.4.6. Pictured are two representative areas of infected cell nuclei, probed with either **(A)** anti-GmSAT1 serum (1:100 dilution) followed by gold conjugated anti-rabbit IgG (1:10 dilution) or **(B)** probed only with the gold conjugated anti-rabbit IgG. Immunogold labelling is marked with arrows. No immunogold labelling was observed in sections probed with gold conjugated anti-rabbit IgG alone.

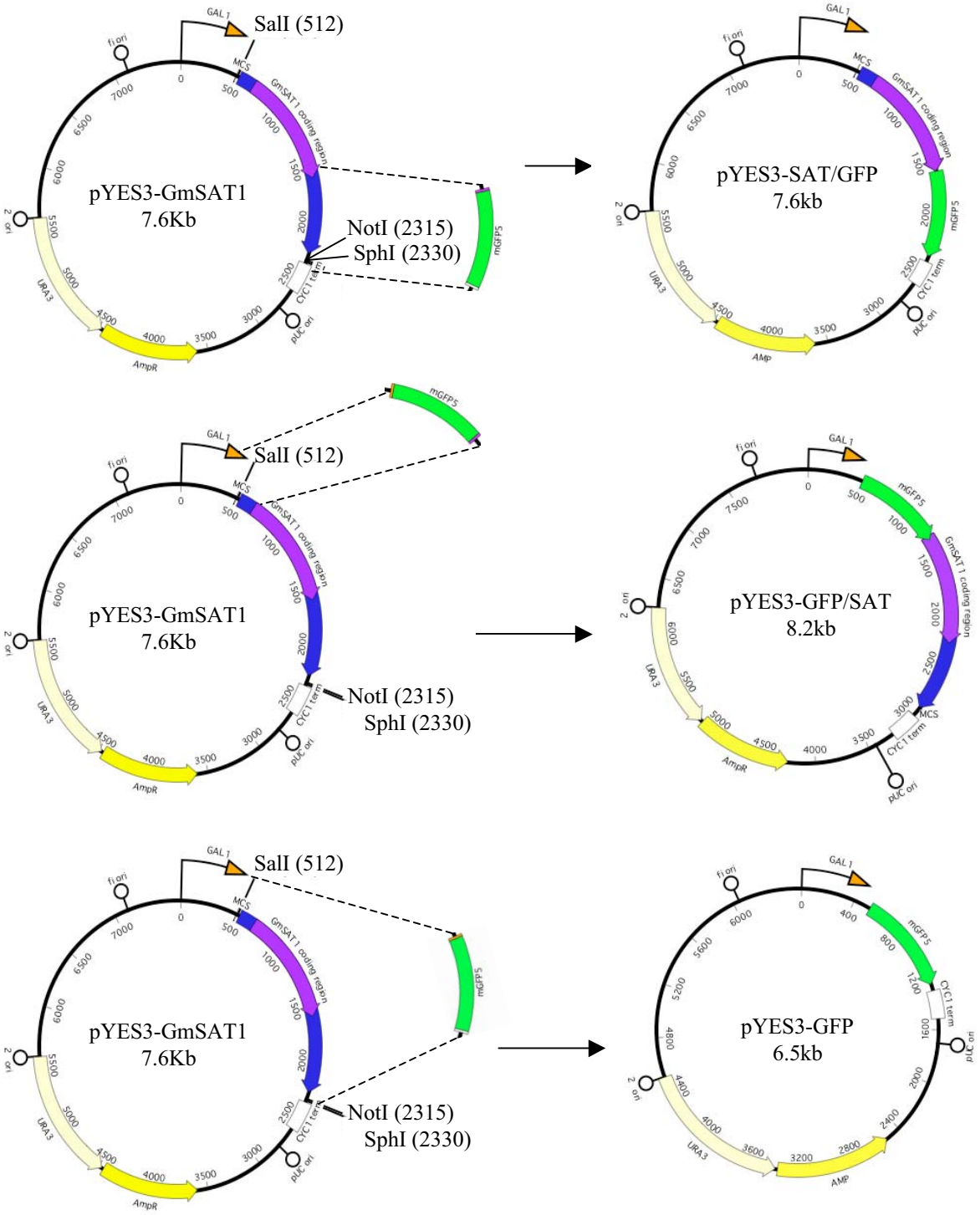



**Figure 3-5. Construction of yeast GFP expression vectors**. *mgfp5* was amplified from pCAMBIA 1302 (www.cambia.org) using primers described in table 3-1 and inserted into the pYES3-GmSAT1 yeast shuttle vector as indicated above using yeast homologous recombination (section 3.4.1). The unique restriction enzyme sites used to digest the vector prior to yeast transformation to generate pYES3-GFP/SAT (*Sal* I), pYES3-SAT/GFP (*Not* I / *Sph* I) and pYES3-GFP (*Not* I / *Sph* I) are indicated. URA3, $Amp^R$ - yeast and bacterial selectable markers. Gal1- Gal1 high expression promoter. Cyc1 term - cyc1 terminator. pUC ori, f1 ori - bacterial origin of replication. 2 ori - 2μ yeast replication origin

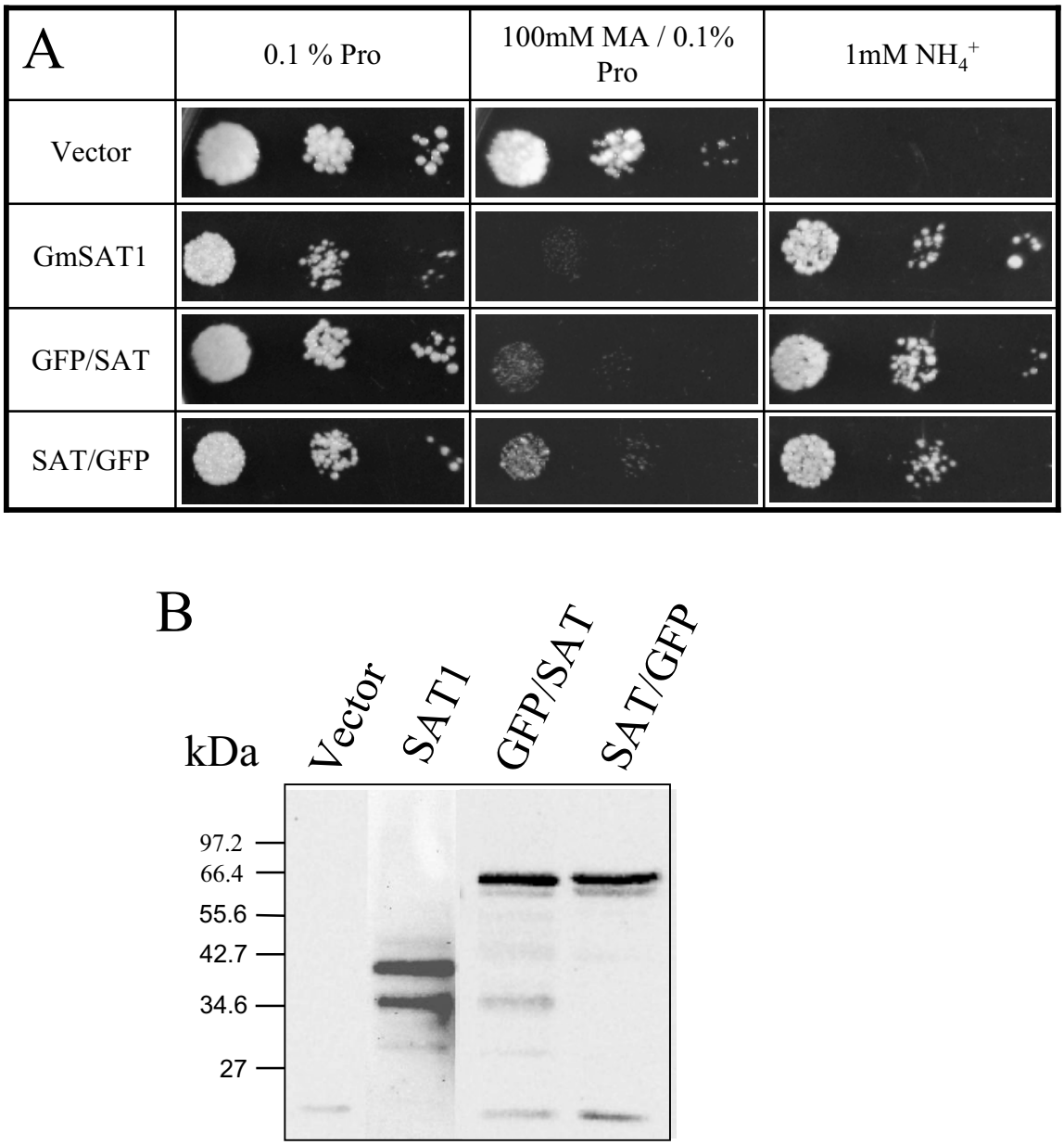



**Figure 3-6. GFP-fusions of GmSAT1 are functional. (A)** 26972c yeast, harbouring the empty pYES3 vector (vector), GmSAT1 in pYES3 (GmSAT1), N- (GFP/SAT) or C-terminally (SAT/GFP) GFP-tagged GmSAT1 in pYES3 were spotted onto solid minimal media plus 2% (w/v) galactose, supplemented with 0.1% proline (Pro), 100mM methylammonium chloride/ 0.1% (w/v) proline (Pro/MA) or 1mM $NH_4Cl$ (1mM $NH_4^+$) **(B)** Total protein from 26972c cells with either empty vector (Vector) or expressing GmSAT1 (SAT1) or the GmSAT1-GFP fusion proteins were subjected to SDS-PAGE, western blotted and probed with anti-GMSAT1 serum. Both the N- (GFP/SAT) and C-terminally (SAT/GFP) GFP tagged GmSAT1 proteins were stably expressed and ran at their expected molecular weight (~ 66 kDa).

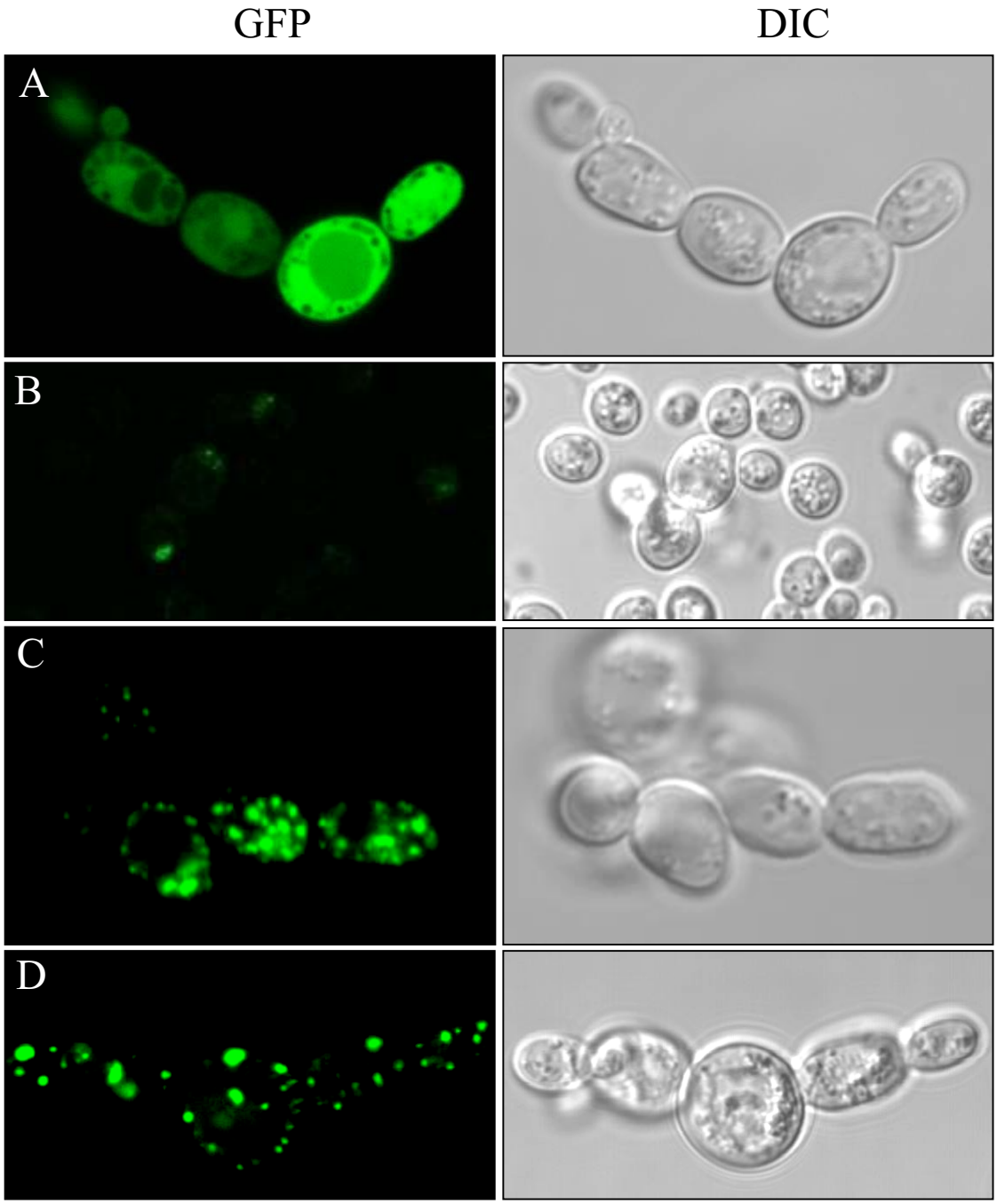



**Figure 3-7. Localisation of GFP expressed alone or fused to the C-terminus or N-terminus of GmSAT1 expressed in yeast.** 26972c yeast were grown in minimal medium supplemented with 0.1% (w/v) proline and 2% (w/v) galactose to induce gene expression under the Gal1 promoter. GFP was visualised using a Leica Spectral Confocal microscope and light pictures taken with differential interference contrast (DIC) microscopy. **(A)** GFP expressed alone was localised throughout the cytoplasm of the yeast. **(B)** GFP signal weakly from the C-terminal GFP GmSAT1 fusion. **(C, D)** GFP fused to the N-terminus of GmSAT1 localises to distinct, punctate vesicles around the periphery of the yeast cell, adjacent or on the plasma membrane.

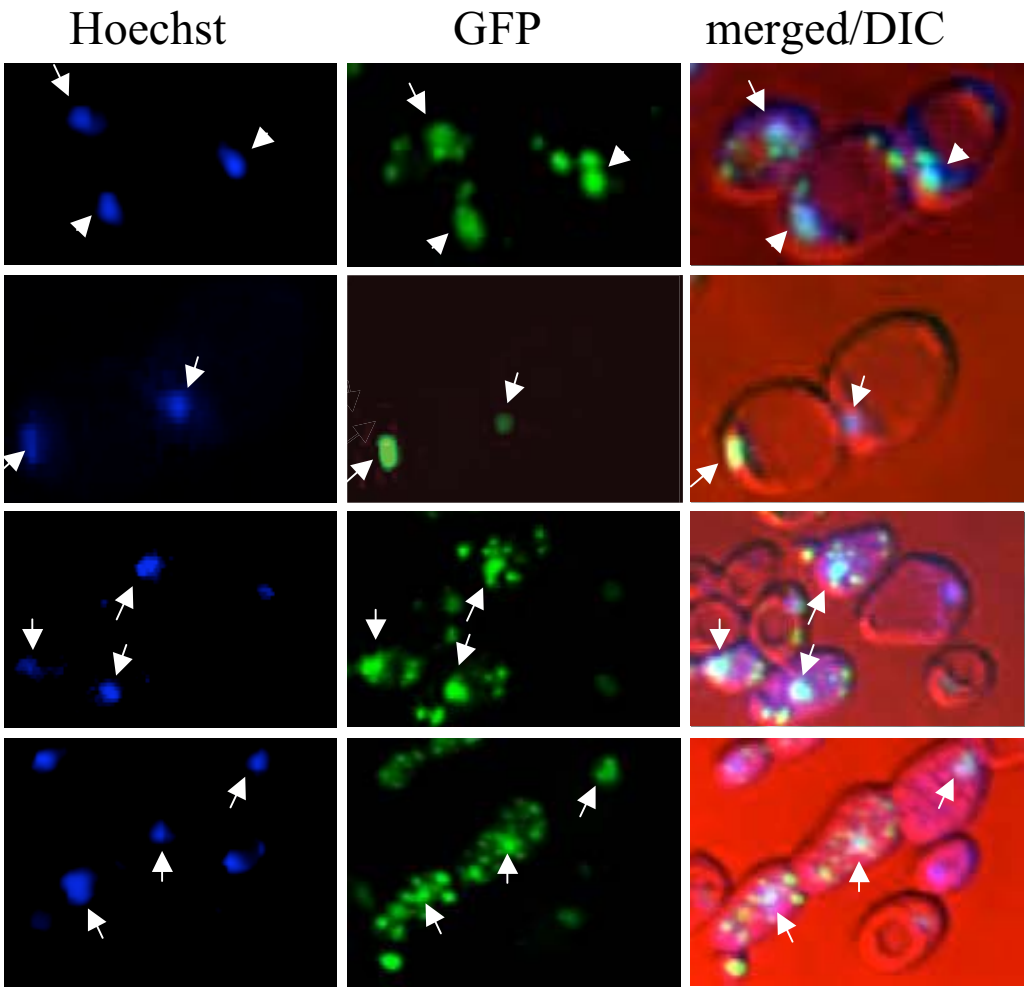



**Figure 3-8. N-terminal GFP-GmSAT1 fusion protein localises to small punctate bodies and the nucleus of yeast.** 26972c yeast were grown in minimal medium supplemented with 0.1% (w/v) proline and 2% (w/v) galactose to induce expression of. GmSAT1 tagged with N-terminal GFP and stained with the vital DNA stain hoechst 33342 as described in section 3.4.6. Yeast were visualised using a Biorad MRC-1000UV confocal microscope using appropriate filters. GFP signal which co-localises with the hoechst stain is marked with arrows.

A

NOTE:
These figures are included on page 3-33 of the print copy of the thesis held in the University of Adelaide Library.

B

**Figure 3-9**. **The Dualsystems membrane protein yeast two hybrid system (taken from www.dualsystems.com). (A)** Two membrane-bound proteins are tagged with either the C-terminal half of the ubiquitin moiety and the artificial transcription factor LexA/VP16 (bait), or the N-terminal half of ubiqutin (prey) . If there is no interaction between the two proteins, the two halves of the ubiquitin are not brought into close proximity and the artificial transcription factor remains bound (left). If there is a specific protein-protein interaction between bait and prey (right), the two halves of the ubiquitin come together and activate ubiquitin-specific proteases. These cleave the artificial transcription factor from the bait and allow its import into the nucleus and transcription of the HIS3 and lacZ reporter genes. **(B)** The pNCW and pCMBV4 vectors allow the N- or C-terminal tagging of a protein of interest with the LexA/VP16 plus the C-terminal half of the ubiquitin moiety (Cub). Low level expression is driven by the cyc1 promoter (Cyc1). The CEN/ARS yeast origin of replication allows the stable propagation of low plasmid numbers (1-2 copies/cell). KanR and LEU2 - bacterial and yeast selectable markers respectively. GmSAT1 and ScMEP3 were amplified using primers described in table 3-1 and cloned in frame into the *Pst* I / *Sac* II restriction endonuclease sites of pNCW. GmSAT1 was recombined into pCMBV4 as described in section 3.4.1

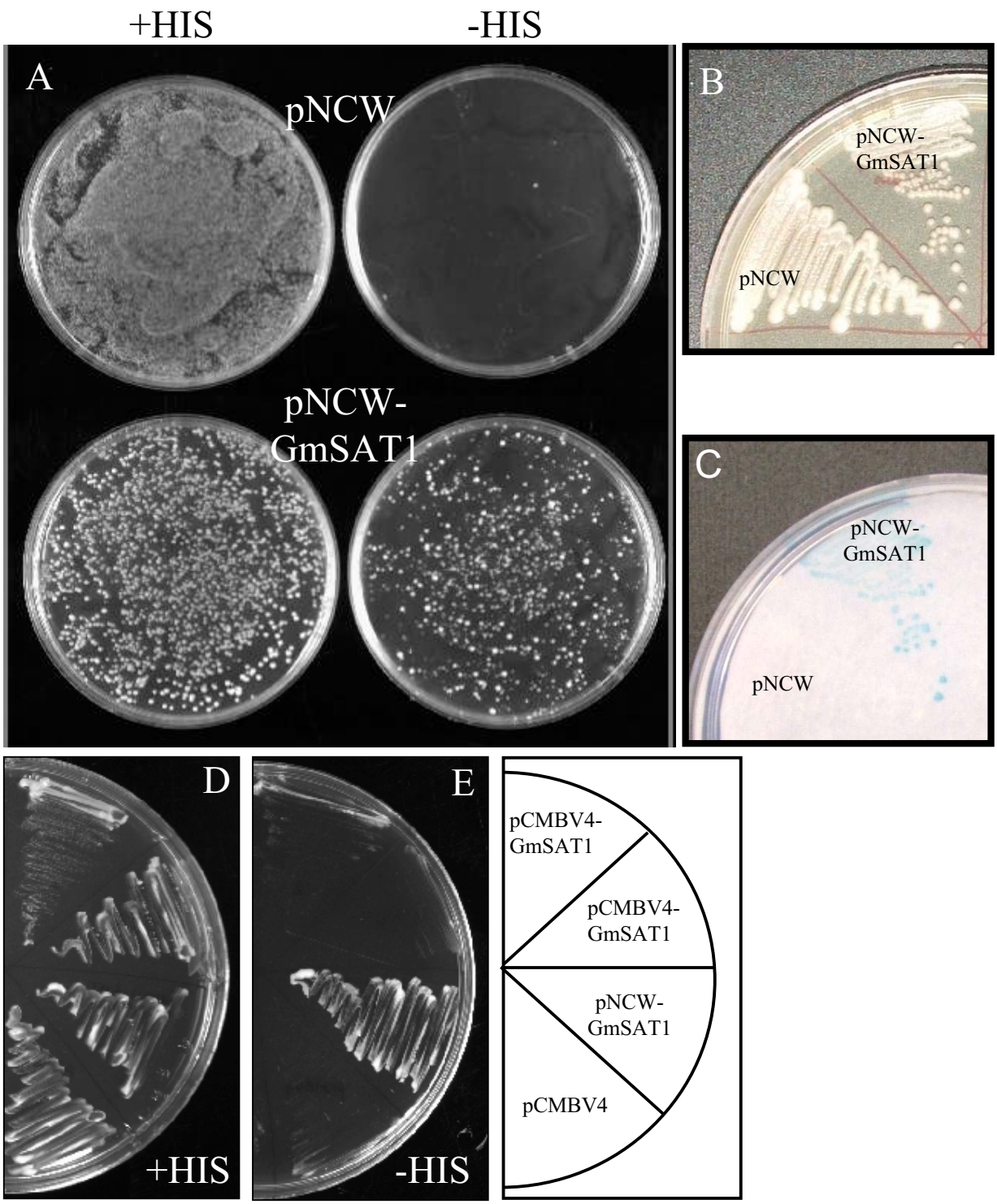



**Figure 3-10. Activation of reporter gene expression in DSY-1 yeast expressing GmSAT1 fused with an N-terminal but not C-terminal artificial transcription factor.** Yeast were grown on solid YNB (w/o amino acids, Difco) plus 2% (w/v) glucose media, supplemented with adenine and tryptophan, plus and minus histidine as indicated. **(A)** The DSY-1 reporter yeast strain was transformed with either empty pNCW vector or pNCW-GmSAT1 and plated on plus and minus histidine media (+HIS, -HIS). Yeast transformed with the pNCW-GmSAT1 construct were able to grow on -HIS medium, indicating activation of the HIS3 reporter gene. **(B)** Colonies from plus histidine plates were restreaked, grown for 48 hours and analysed for lacZ activity (blue colour, **(C)**) as described in section 3.4.4. DSY-1 harboring pNCW-GmSAT1, empty pCMBV4 or pCMBV4-GmSAT1 were streaked on media with **(D)** and without **(E)** histidine as indicated. GmSAT1 with the C-terminal artificial transcription factor (pCMBV4-GmSAT1) did not activate reporter gene expression (no growth on –HIS).

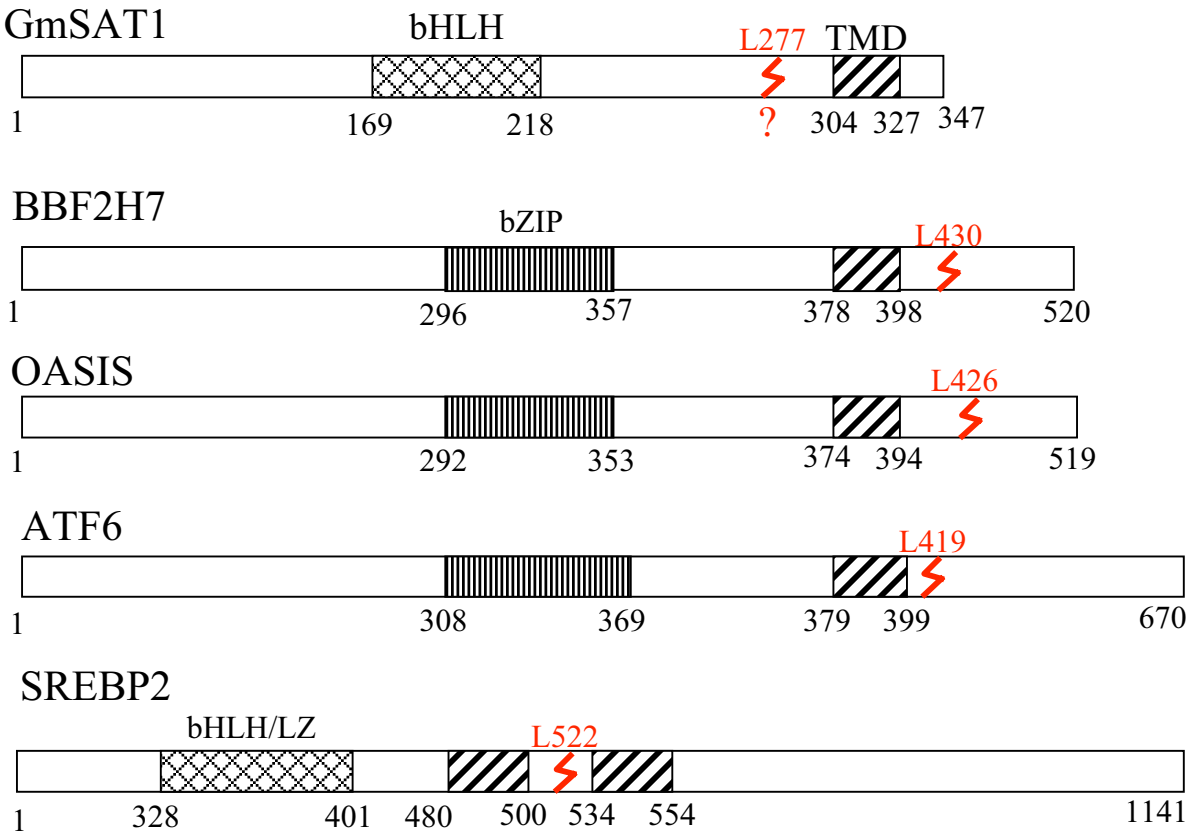



**Figure 3-11. Comparison of the protein structures of GmSAT1 with mammalian membrane-bound transcription factors cleaved by the Site-1 protease.** The basic Helix-Loop-Helix (bHLH), basic leucine zipper (bZIP), and bHLH/leucine zipper (bHLH/LZ) transcription factor domains and predicted transmembrane domains (TMD, diagonal line box) are marked. The subtilisin-like site-1 endoprotease cleaves the bottom four transcription factors after the leucine residue designated in red, followed by a second cleavage within the transmembrane domain to liberate the transcription factor domain. The putative GmSAT1 site-1 protease cleavage site (L277) is also marked. Accessions (in order): AAC32828, NP_919047, Q9Z125, AAB64434, NP_004590

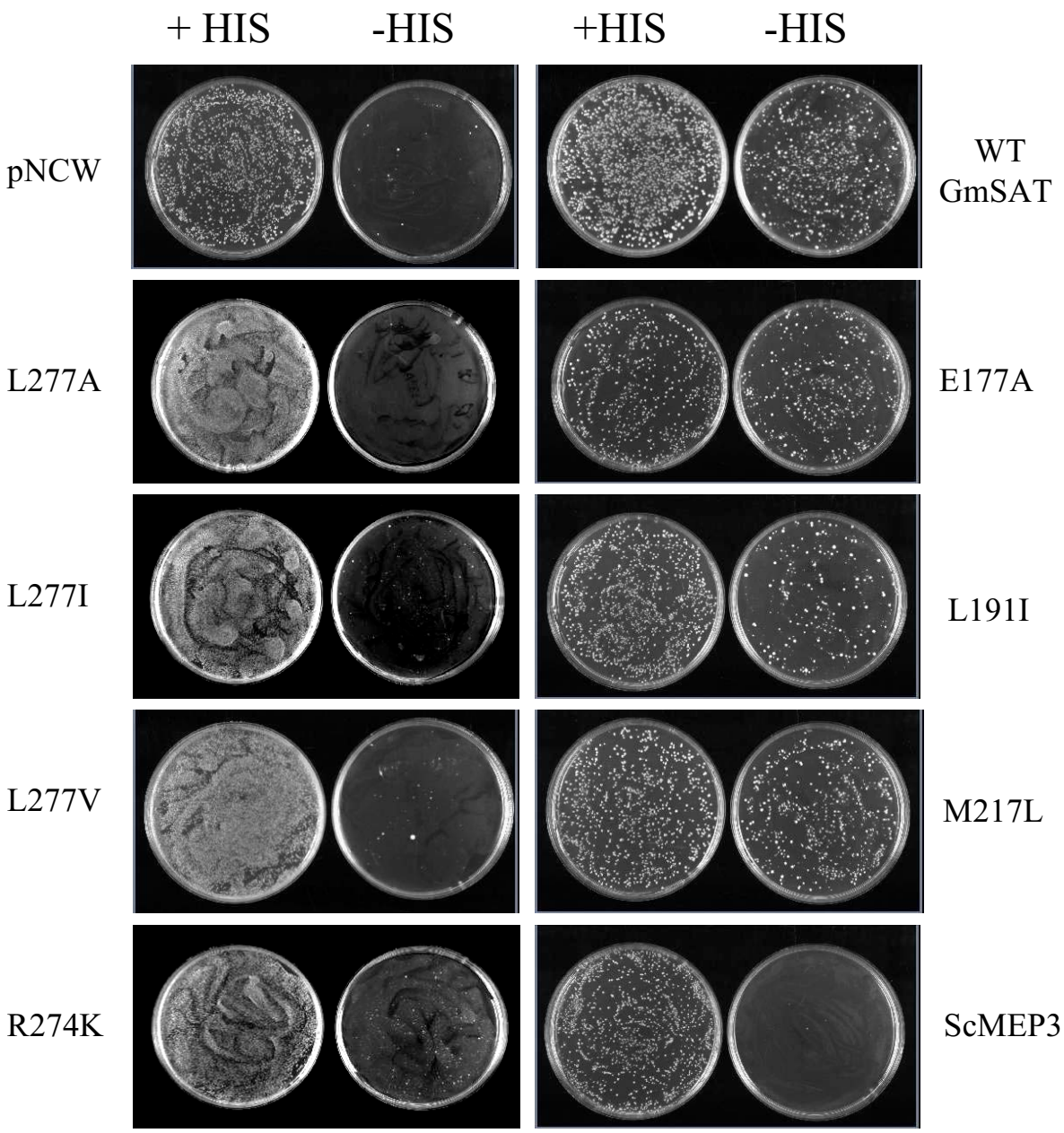



**Figure 3-12. The effects of various amino acid substitutions in GmSAT1 on HIS3 reporter gene expression.** The reporter yeast strain DSY-1 was transformed with either empty pNCW vector, pNCW with wild-type or mutant GmSAT1 (as indicated), or pNCW-ScMEP3 and plated on minimal media plus or minus histidine (+HIS and -HIS respectively) supplemented with adenine and tryptophan, minus leucine for selection of transformants. When the VP16/LexA transcription factor is fused to the *bona fide* membrane bound protein ScMEP3, there is no self activation evident (no growth on histidine deficient media).

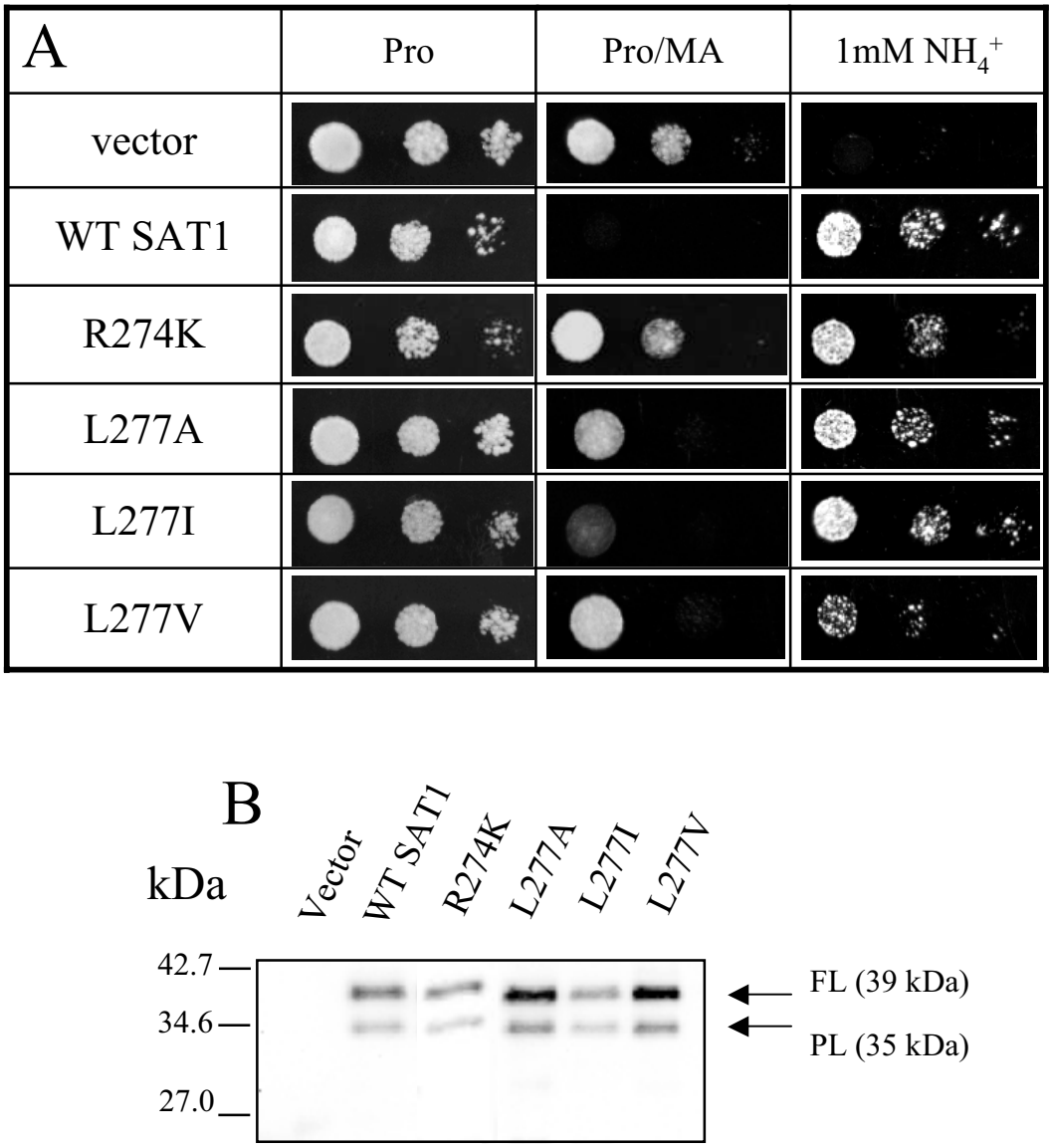



**Figure 3-13. Effects of amino acid substitutions in the putative proteolysis site of GmSAT1. (A)** GmSAT1 with amino acid substitutions in the putative proteolytic cleavage site were expressed under the Gal1 promoter in the yeast strain 26972c. Yeast were spotted onto minimal media supplemented with 2% (w/v) galactose and 0.1% (w/v) proline (Pro), 0.1% (w/v) proline/100mM methylammonium chloride (Pro/MA) or 1mM $NH_4Cl$ ($NH_4^+$). **(B)** Total protein from 26972c cells, grown in YNB plus 2% (w/v) galactose, expressing GmSAT1 with the indicated amino acid substitutions were subjected to SDS-PAGE, western blotted and probed with anti-GMSAT1 serum. The mutations do not affect the appearance of the 35 kDa, putative proteolysis product. FL, full length GmSAT1 PL, proteolysis product of GmSAT1

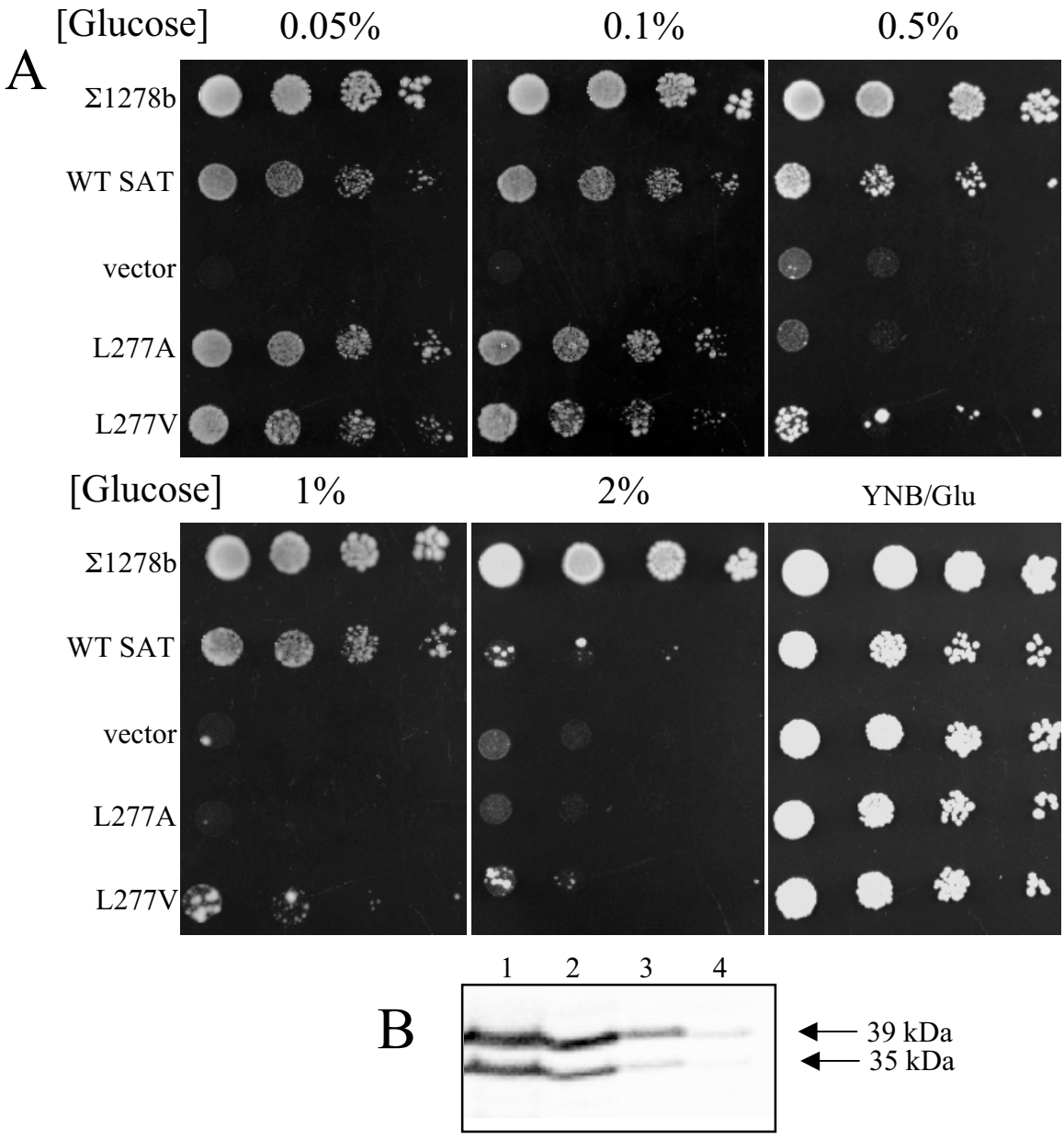



**Figure 3-14. Modulating GmSAT1 expression levels reveals differences between wild-type and L277 mutants in their ability to complement 26972c on 1 mM $NH_4^+$.** **(A)** The wild-type yeast strain Σ1278b and the ammonium transport yeast mutant 26972c harbouring empty pYES3 (vector), or pYES3 with wild-type (WT SAT) or mutant GmSAT1 (L277A and L277V), were grown to mid-log phase in YNB (w/o amino acids) plus 2% (w/v) glucose, washed twice, and a 10-fold dilution series spotted onto minimal medium with 1mM $NH_4Cl$ as the sole nitrogen source, plus 2% (w/v) galactose and the various glucose concentrations (0.05% to 2% ) indicated. Yeast were also spotted on YNB (w/o amino acids) plus 2% (w/v) as a loading control. **(B)** Equal amounts of total protein samples from yeast expressing wild-type (lanes 1 and 3) or L277A mutant GmSAT1 (lanes 2 and 4) grown in YNB plus 2% (w/v) galactose, plus either 0.1% (lanes 1 and 2) or 0.75% (lanes 3 and 4) (w/v) glucose were subjected to SDS-PAGE, western blotted and probed with anti-GmSAT1 antiserum.