CHAPTER-II

EXPRESSION OF GUS GENE UNDER DIFFERENT PROMOTERS IN S. CEREVISIAE AND A. TUMEFACIENS

Expression of GUS gene under different promoters in *S. cerevisiae* and *Agrobacterium tumefaciens*:

After studying expression of GUS gene under different promoters in plants it appeared that At2S3 promoter is not so specific as it was envisaged before because it has been made to behave as an active promoter even in the ABA treated leaves where leaves without treatment showed very little or no activity. Moreover, a 16KDa protein has also been implicated to act as one of the transacting factors to regulate 2S3p in tobacco seeds. Further, a denovo synthesis of 16 KDa factor has been shown after the treatment of transgenic tobacco leaves (Chapter I).

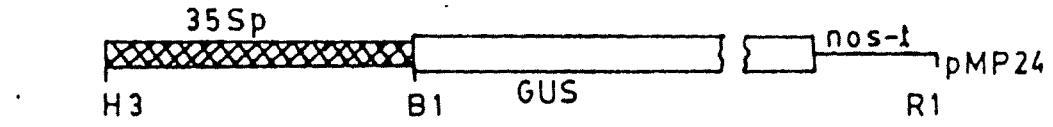
Saccharomyces cerevisiae, being an evolutionary conserved organism, is widely used as a model system to unravel the complex eukaryotic regulation of gene expression and the results obtained could be extrapolated to explain many finer regulations of higher eukaryotes. In fact, it has been reported from this laboratory, that 2S gene under its own promoter is expressed and processed in yeast (Pal and Biswas, 1995) but expression level is low (0.032%) and due to complex posttranslational processing of the seed protein, the assay of promoter activity becomes difficult. So, an alternative approach of analysing the promoter activity with reference to the reproter gene, GUS has been used.*S. cerevisiae*, like plants lacks endogenous GUS activity and hence detection of GUS enzyme provides a measure of gene expression with very low background activity.

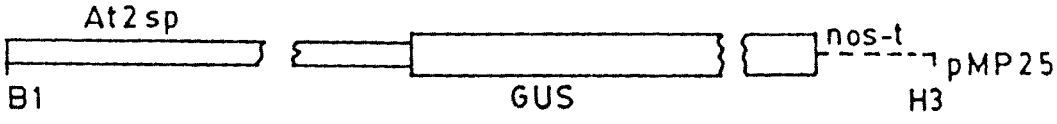
In case of *Agrobacterium*, though there are some occasional reports of background GUS activity from some GUS plasmids arising due in part to read-through transcription from the lacZ transcript of pBIN19 into the GUS coding region, followed by translational initiation at the GUS initiator; *Agrobacterium* and *Rhizobium* without these plasmids shows little, if any, detectable GUS activity. The 2S3 promoter activity has been studied with respect to GUS gene expression in *Agrobacterium* and yeast. Furthermore, the GUS reporter gene is used to compare the activity of At2S3p with an endogenous yeast promoter, PyKp and a plant constitutive promoter, CaMV35S. Moreover, specific transacting factor(s) like the ~16KDa protein as detected in the mid maturation phase of the seeds as well as in the ABA treated leaves of the tobacco plant, in case of both yeast and *Agrobacterium*, has been looked for.

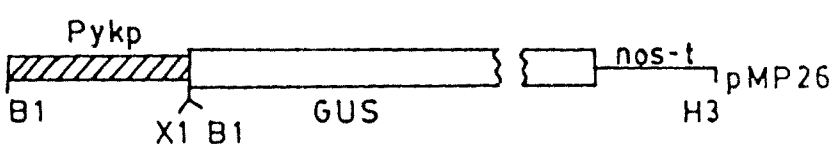
Monitoring promoter strength using GUS as a Reporter gene:

In order to compare the efficiency of At2S3 promoter with the Pyk promoter and CaMV35S promoter, three different vectors viz. pMP24, pMP25 and pMP26 (Fig.2.1) were constructed as mentioned under section, Materials and Methods. In those constructs, such as pMP24 and pMP25,the translational initiator ATG is the same as it is in pBI 121 (Jefferson, 1987). In pMP26, the translational initiator of PyK was kept as the first ATG (Burke et al, 1983) and also the PyKp was taken as a 0.45Kb DNA fragment including both the TATA and CAAT boxes. All the three constructs viz. pMP24, pMP25 and pMP26 were made in the yeast integrating plasmid YIP5 (Fig. 2.2; Struhl et al., 1979).

Fig. 2.1: Vector construction. 35S$_P$ cauliflower mosaic virus 35S promoter; PyK$_P$ pyruvate kinase promoter; GUS, β-D glucuronidase coding sequence of E.coli; nos-t, nopaline synthase terminator; BI, BamHI; NI, NcoI; RI, EcoRI; H3, HindIII; the thicker box represents the At2S3 coding sequence. The details of the construction procedure is described in the Materials and Methods section.







At2Sp ⇨ 1·5 Kb ; GUS ⇨ 1·9 kb
35Sp ⇨ 1·0 Kb ; Pykp ⇨ 0·45 kb
nos-t ⇨ 0·3 Kb

Fig 2·1

Fig. 2.2: Detailed restriction map of yeast integrating plasmid (YIP5) used as a vector for construction of pMP24, pMP25 and pMP26. Ap and Tc represent ampicillin and tetracycline resistance gene respectively. ORI is the origin of replication.

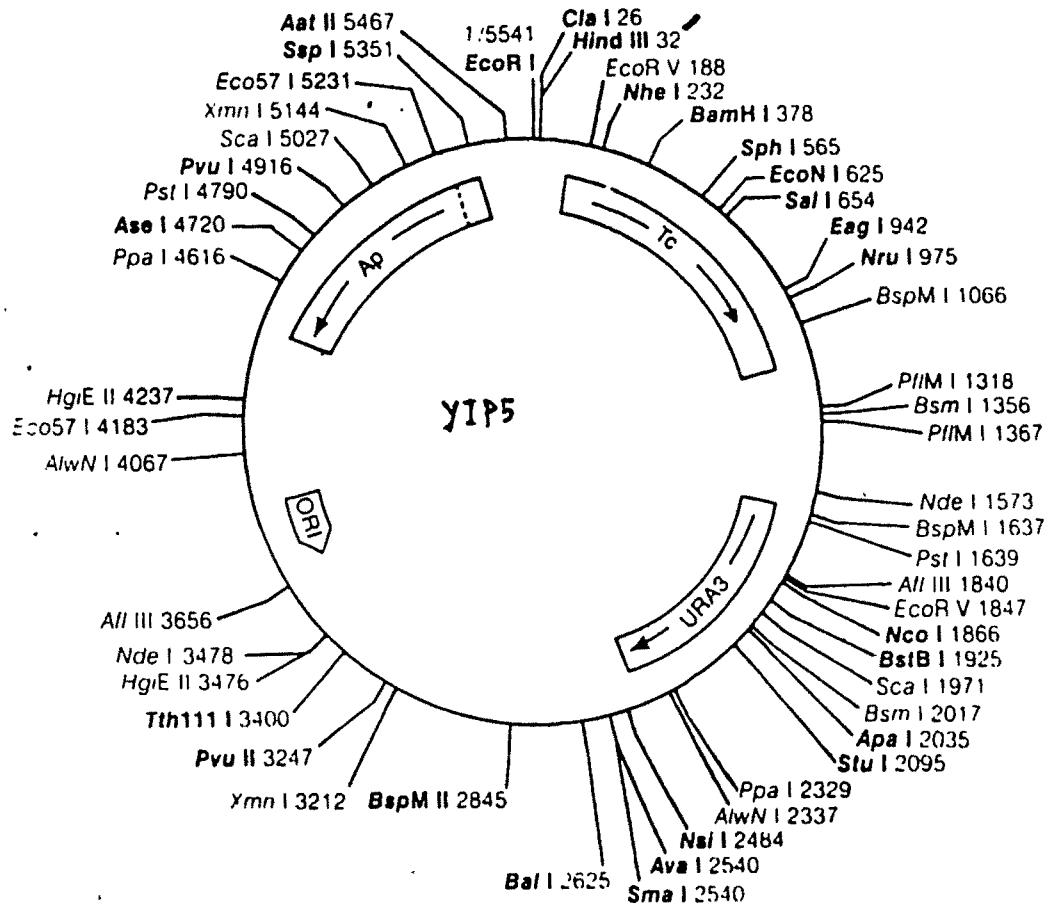

YIP5
Ap
Tc
ORI
URA3
Aat II 5467
Ssp I 5351
Eco57 I 5231
Xmn I 5144
Sca I 5027
Pvu I 4916
Pst I 4790
Ase I 4720
Ppa I 4616
HgiE II 4237
Eco57 I 4183
AlwN I 4067
All III 3656
Nde I 3478
HgiE II 3476
Tth111 I 3400
Pvu II 3247
Xmn I 3212
BspM II 2845
Bal I 2625
Sma I 2540
Ava I 2540
Nsi I 2484
AlwN I 2337
Ppa I 2329
Stu I 2095
Apa I 2035
Bsm I 2017
Sca I 1971
BstB I 1925
Nco I 1866
EcoR V 1847
All III 1840
Psi I 1639
BspM I 1637
Nde I 1573
PflM I 1367
Bsm I 1356
PflM I 1318
Nru I 975
Eag I 942
Sal I 654
EcoN I 625
Sph I 565
BamH I 378
Nhe I 232
EcoR V 188
Hind III 32
Cla I 26
EcoR I 5541

Fig2-2

Transformations of the three constructs (pMP24, pMP25 and pMP26) were tested for their genomic integration and unit copy per cell for comparing promoter strengths. The promoter strengths were measured with reference to the reporter enzyme activity as quantitated by the fluorometric assay (Table 2.1). It is apparent from the data that At2S3p is 45 fold less active than the PyKp and the CaMV35S is very inefficient and 32 fold less active than At2S3p. Northern blot analysis using total RNA isolated from pMP24, pMP25 and pMP26 transformants and taking GUS gene as a probe also reaffirmed the fluorometric observation. Since, it is clear from Fig. 2.3, that there is very strong GUS gene transcript generated in case of pMP26 (lane 2, Fig.2.3) compared to pMP25 (lane 1, Fig.2.3), whereas in case of pMP24 there is hardly any transcript visible (data not shown) which is expected as 35S promoter is 45 fold less active than At2S3p or in other words 77 fold less active than PyKp.

Determination of transcription start point (tsp):

Whether the plant promoter was correctly recognised by the yeast, the tsp of the GUS gene transcript under different promoters by primer extension analyses (Sambrook et al., 1989) was determined. Experiments with total RNA isolated from pMP25 transformants indicated that the transcription of GUS was initiated at a point of the pAt2S3 equivalent to about 36 nucleotide upstream from A of the initiation codon ATG of At2S3, which is also the tsp of GUS under the same promoter in plant (Fig. 2.4 , lanes 1 and 2). In transformed tobacco plants, the tsp of 2S3 was found to occur at the same point, which is

Fig. 2.3: Northern blot hybridisation of GUS gene transcript in case of pMP26, pMP25 transformed yeast cells. Total RNA (20 µg) was seperated in 1.2% formaldehyde agarose gel and hybridised with ^{32}P labelled GUS gene as a probe. Lanes 1, and 2 correspond to pMP25, pMP26 transformed yeast cells respectively.

Fig. 2.4: Primer extension experiment to determine the transcription start point (tsp). Total RNA of yeast transformants and plant as noted below were annealed with 10^4 cpm of gamma ^{32}P labelled 22-mer oligo 5' GATTTCACGGGTTGGGGTTTCT 3' at 28 °C with 80% formamide. The oligo was annealed 14 base downstream of A of initiation codon ATG of GUS. 90 µg of total RNA from pMP24 and 30 µg of total RNA from each of pMP25, pMP26 and tobacco plant (transformed with pMP17 construct) were used. Total RNA was lithium chloride precipitated, treated with RNase free DNase to remove contaminating DNA. Reactions were carried out with 7.5 U AMV reverse transcriptase at 42 °C. Control experiments were carried out with equal amount (50 µg) of total RNA from untransformed yeast and plant and no visible band was discernible in those two lanes. Lanes 1, 2 and 4 correspond to overnight exposure. Exposure of these upto 5 days did not show any other band in those lanes. Arrowws at right represent transcription start points. 35-mer corresponds to the position of oligos of 35 nucleotides.

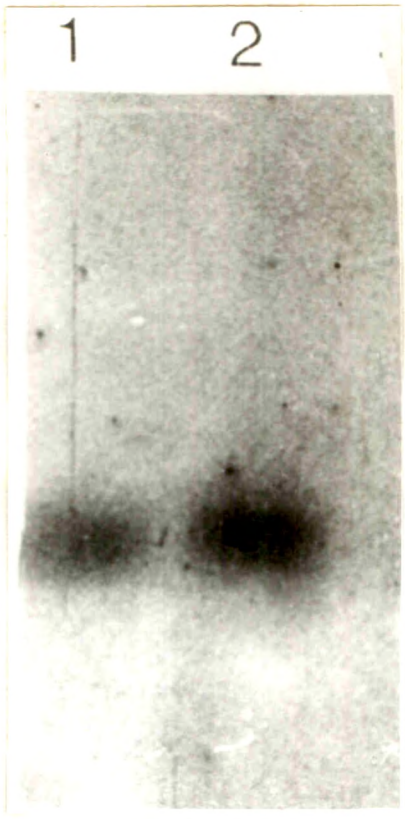


Fig. 2.3

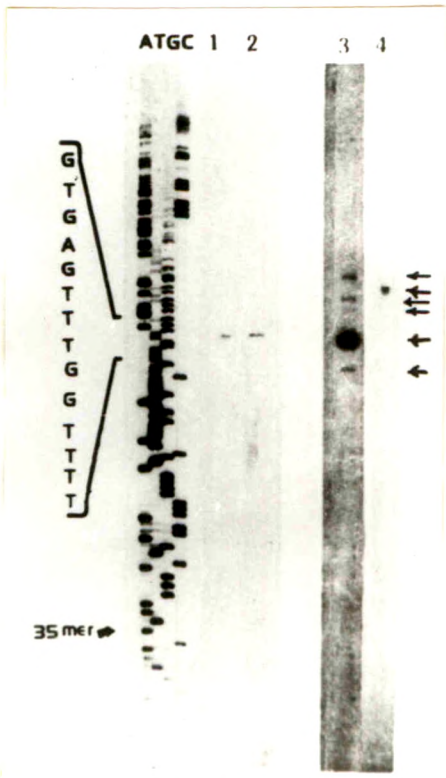


Fig. 2.4

Table 2.1

Efficiency of different promoters as assayed by GUS activity in *S. cerevisiae* under experimental conditions

Promoter	(GUS activity expressed in nmoles of 4-MU produced / mg of protein / hr)
CaMV35S	80
At2S3	2490
PyK	111970

NcoI linearised pMP24, pMP25, pMP26 and YIP5 were introduced into yeast cells to integrate into the Ura3 gene. Transformants were selected on Uracil minus agar medium. The transformants of each construct having contained a single copy of the insert was determined in case of yeast. Cells were grown at 30°C in YEPD medium upto O.D 2 (650 nm) and resuspended in the concentration of 10^{10} cells/ml of GUS extraction buffer. 40 µg of crude protein from each of the three transformants along with a control was taken for assay of GUS activity using 4-methyl Umbelliferyl β-D-Glucuronide as substrate. Nontransformed cells showed very little GUS activity (30 nano moles of 4-MU produced/mg of protein /h) which have been treated as background and substracted to obtain the values as shown in the table. A mean value of three different experiments was given (a variation was 10 %).

Fig. 2.5: Restriction digestion to confirm the clone bearing 0.94 Kb At2S3 promoter in YIP5. Digestion with EcoRI and HindIII yielded two bands of molecular weights 5.4 Kb and 3.3 Kb respectively (lane 2). Lane 1 is the lambda HindIII digested M.W. marker. Sharp band is not visible probably due to the contaminating DNase.

Fig. 2.6: Northern blot hybridisation to determine GUS gene transcript in yeast cells transformed with constructs containing GUS gene under full length (1.5 Kb) vis-a-vis truncated (0.94 Kb) At2S3 promoter. Total RNA (20 µg) were separated in 1.2% formaldehyde agarose gel and hybridised with ^{32}P labelled GUS gene as a probe. Lanes 1 and 2 correspond to GUS gene transcript under 1.5 Kb At2S3p and 0.94 Kb At2S3p respectively.

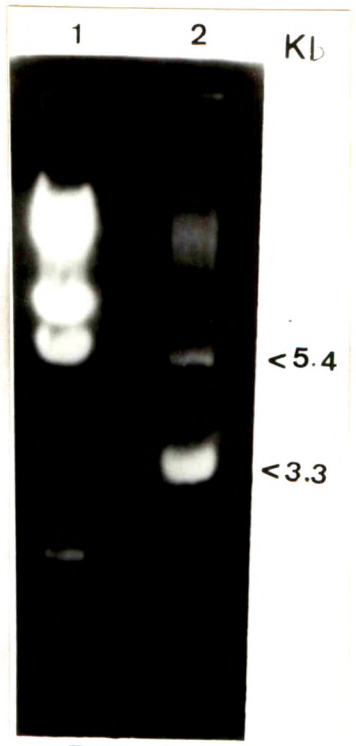


Fig 2.5

1 2

Fig. 2.6

very similar to that of the napin gene (Jensen et al., 1986). Cramer et al, 1985 reported the different tsp of phaseolin mRNA from its own promoter (though some of the minor tsp remained same) in *S. cerevisiae* than that in bean whereas same tsp of zein in *S. cerevisiae* as in plant was reported by Coraggio et al. (1986) using GAL-UAS hybrid promoter. Analysis with the transcript from pMP24 transformants indicated that the transcripts were initiated from one major and several minor points none of which (Fig. 2.4, lane 3) is the tsp in plant (Pobjecky et al., 1990). The RNA from the untransformed cells was used in the experiment as a control and no extended product was recorded (data not shown). The primer extended product of total RNA corresponding to pMP26 transformants showed up with the tsp similar as (Fig. 2.4, lane 4) reported earlier for the PyK gene (Burke et al., 1983). Therefore it is apparent that for GUS gene, the pAt2S3 can effectively be recognized by *S. cerevisiae*.

Cloning of 0.94Kb At2S3 promoter into YIP5:

In the previous chapter, it has been shown that 0.94Kb At2S3 promoter could retrieve 90% activity of the full length 1.5Kb At2S3p in tobacco plant. In order to see what happens to the 0.94Kb At2S3p fused to GUS gene when introduced into YIP5 and to draw an analogy with respect to the full length 1.5Kb At2S3p fused to GUS gene when they were introduced separately into yeast.

In order to clone 0.94Kb At2S3 promoter fused to GUS in YIP5, GUS gene under 0.94Kb At2S3p was digested with BamH1 and HindIII and the

0.94kb At2S3 promoter fragment was purified. The 2.2Kb GUS coding sequence was purified from pMP8 (GUS in pBS as shown in (Fig.1.2) after digesting it with BamH1/EcoR1. Similarly, YIP5 (Yeast Integrating Plasmid) was digested with EcoR1/HindIII and the large fragment was purified. The three fragments were then ligated and transformed into *E.coli* DH5α and the transformants were confirmed by minipreparation of the plasmid DNA followed by restriction digestion for the selection of the right clone (Fig.2.5). One of the positive clones was taken for large scale plasmid preparation and the plasmid DNA was purified. Purified DNA was digested with EcoR1/HindIII and expected band profiles were obtained as shown in the figure 2.5. The lane 2 corresponds to the EcoR1/HindIII digested DNA as there are two bands of expected molecular sizes, 5.4Kb and 3.3Kb.

The two constructs viz. 1 5.kb and 0.94Kb 2S3p both containing GUS gene in YIP5 were then introduced into yeast cell, BJ2168 following standard transformation protocol & then GUS gene expression was monitored both fluorometrically and by northern blot analysis. Fluorometric study indicates that 0.94Kb At2S3p could retrieve almost 80% of the activity of the full length 1.5Kb At2S3 promoter. Northern blot analysis also confirmed that the GUS activity, fluorometrically determined is proportional to the extent of mRNA produced. Because in northern hybridisation the difference in the signals generated in lane 1 and 2 corresponding to the mRNA transcripts arise from *S. cerevisiae* transformed with constructs containing GUS gene under 1.5Kb 2S3p & 0.94Kb 2S3p respectively do not vary significantly (Fig. 2.6).

Determination of transacting factor in *S. cerevisiae*:

In order to determine any specific transacting factor that might have interacted with At2S3 promoter, when it was introduced into yeast, Southwestern experiment taking nuclear extract isolated from *S. cerevisiae* was performed. In the Southwestern blot, only one signal in the ~16kDa region (Fig. 1.13) was detected. This matches exactly with one of the signals generated in case of nuclear extracts of mid maturation phase of the seeds of the tobacco plant.

Expression of GUS gene under 1.5kb vis-a-vis 0.94kb At2S3 promoter in *Agrobacterium tumefaciens*:

Agrobacterium tumefaciens as one of the systems was used to see the GUS gene expression under At2S3 promoter. *Agrobacterium tumefaciens* is a gram negative soil bacterium that causes crown gall disease in dicotyledonous plants. So, it's long association with plants in nature was looked upon as a major trait for being considered as a likely candidate to see its ability to recognize a seed specific plant promoter like At2S3. Vectors containing GUS gene fused to full length 1.5 Kb At 2S3p & 0.94 Kb At2S3p which were used to see GUS gene expression in tobacco plant were used. The GUS gene expression in pMP17 transformed *Agrobacterium* was first assayed fluorometrically. It is apprent from Table 2.2 that GUS gene expression under 1.5 Kb At2S3 promoter in *Agrobacterium* could be compared with the same in tobacco plant. In *S.*

cerevisiae, though the GUS gene activity under 1.5 kb At2S3p was about 30% of what has been recorded in the case of tobacco and *Agrobacterium*, yet it is very significant. A comparative study of GUS gene expression under 1.5 Kb At2S3p against 0.94 Kb At2S3p in tobacco, yeast and *Agrobacterium* is given in Table 2.2 below.

TABLE 2.2

Expression of GUS gene under full length (1.5 Kb) vis-a-vis truncated (0.94 Kb) At2S3 promoter in Tobacco, yeast and *Agrobacterium:*

System	1.5 Kb At2S3p (GUS activity expressed in n moles of 4-MU produced/mg of protein/hr	0.94 Kb At2S3p
Tobacco	7820	6970
Yeast	2480	1960
Agrobacterium	7492	6750

Again, vector containing GUS gene fused to 0.94kb At2S3 promoter when introduced into *Agrobacterium*, it could restore ~90% activity of the full length 1.5 Kb At2S3p like in tobacco. Fluorometric as well as northern blot (Fig 2.7) analyses show similar trend. There is no sharp difference in the GUS gene transcript from *Agrobacterium* transformed with constructs containing GUS gene under 1.5 Kb At2S3 promoter and 0.94 Kb At2S3 promoter respectively (lane 1 and 2, Fig. 2.7).

Even histochemical analysis of GUS gene product under 1.5 Kb At2S3 promoter against 0.94 Kb At2S3 promoter reaffirms this observation. In Fig. 2.8, lanes 1 and 2 indicate the colour formation due to β-Glucuronidase (GUS) dependent cleavage of X-gluc (substrate of GUS) when GUS was placed under full length 1.5 Kb At2S3 promoter and 0.94 Kb At2S3 promoter respectively. There is no significant difference in the intensity of colour formed in two cases. Lane 3 in Fig. 2.8 was used as a control in which GUS gene was under anti oriented full length 1.5 Kb At2S3 promoter. So, as expected there is no expression of GUS gene and subsequently no colour is developed.

Detection of transacting factor in *Agrobacterium*:

A ~16kDa factor in the nuclear extract of mid maturation phase of the seeds, leaves of the tobacco pretreated with Abscisic acid and in *S.cerevisiae* has already been detected. It is interesting to see whether any such factor in the whole cell extract of *Agrobacterium* is present or not. When Southwestern analysis was performed with the *Agrobacterium* cell extract, in addition to many factors of higher mol. wt., similar ~16kDa factor was also lighted up and it matches well with the ~16kDa band detected in the *S. cerevisiae* (lane 1 and 2, Fig. 2.9). It seems that a similar 16kDa factor might have a role in expressing the GUS gene under At2S3 in *A. tumefaciens*.

Fig. 2.7: Northern blot hybridisation to determine GUS gene transcript in *Agrobacterium* cells transformed with constructs containing GUS gene under full length (1.5 Kb) vis-a-vis truncated (0.94 Kb) At2S3 promoter. Total RNA (20 μg) were separated in 1.2% formaldehyde agarose gel and hybridised with ^{32}P labelled GUS gene as a probe. Lanes 1 and 2 correspond to GUS gene transcript under 1.5 Kb At2S3p and 0.94 Kb At2S3p respectively.

Fig. 2.8: Histochemical detection of the GUS gene product in *Agrobacterium* cell extract transformed with GUS gene under full length (1.5 Kb) At2S3p (left), GUS gene under truncated (0.94 Kb) At2S3p (middle) as well as anti oriented GUS gene under 0.94 Kb At2S3p (right). Cell extract from each of the transformed cell was incubated with X-gluc (100 μg/ml) at 37°C overnight. A little amount of K^+ ferricyanide/ferrocyanide was also added to enhance colour formation.

Fig. 2.7

Fig. 2.8

Fig. 2.9: Southwestern blot to determine 16 KDa factor in *Agrobacterium* cell extract. Lane 2 corresponds to *Agrobacterium* and lane 1 corresponds to yeast run as a control. One of the bands in *Agrobacterium* lane (indicated by arrowhead) matches with the band around 16 KDa region in yeast lane.

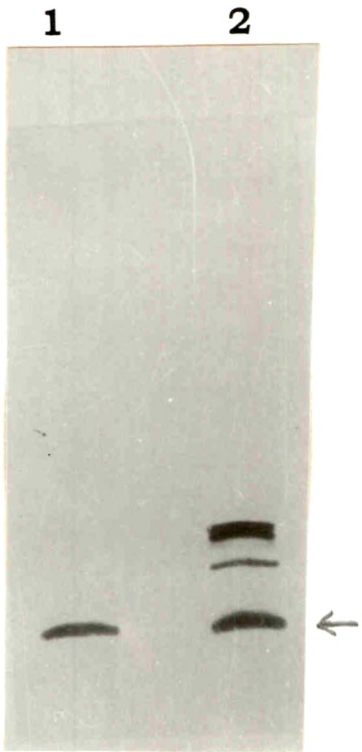


Fig. 2.9