B. PLANT TRANSFORMATION AND GENE EXPRESSION

1. Plant transformation

Tobacco plant was transformed using co-cultivation method of *Agrobacterium* carrying appropriate construct. Small green plantlets started regenerating on selection medium containing either kanamycin (100-200 mg/L) or hygromycin (30 mg/L) after 3 - 4 weeks co-cultivation (Figure 24A). There were no signs of regeneration from untransformed leaf discs (Figure 24A). After 4 weeks, the healthy shoots were transferred to fresh tissue culture bottles containing RM medium and selection agent for further growth and establishment of roots system (Figure 24B). All the green plants that were growing in the presence of selection medium were transferred to soil containing pots for seed setting (Figure 24C-D). At least 10 – 30 independently transformed plants were produced for each construct. To analyse the progeny, seeds were germinated on RM medium containing selection agent, scored the regeneration pattern (Figure 24E) and checked GUS expression at seeds and seedling (Figure 24F). Transgene expression analysis was carried out using both T0 and T1 generation plants.

For rice transformation, embryogenic callus obtained from the scutellar region of the germinating seeds following gene gun method. After transformation using the appropriate construct, bombarded calli were transferred to selection agent (hygromycin (30 mg/L) containing RM medium supplemented with 2 mg/L 2,4-D. After 3-4 weeks most of the calli turned to brown or black in colour. After 4 weeks, white calli started appearing on the selection medium (Figure 25A). The putative transgenic calli was multiplied in the presence of hygromycin (30 mg/L) for another period of 3-4 weeks (Figure 25B). The vigorously growing calli were transferred to hormone free RM medium for regeneration of shoots (Figure 25C). Green plantlets started appearing within 2-4 weeks on the regeneration medium (Figure 25D-E). At least 10 - 15 independently transformed plants were regenerated for each construct. After one month, well established plants with well developed root system were transferred to soil and grown in the greenhouse for seed setting (Figure 25F).

For tomato transformation, seedlings were germinated in culture bottles after surface sterilization (Figure 26A). From the germinated seedlings, cotyledons were first precultured on MS medium containing 2 mg/l kinetin for two days. This was done to eliminate the soft cotyledons that get damaged during dissection. While the damaged cotyledons respond poorly and the intact cotyledons respond well for *Agrobacterium* mediated transformation. Cotyledons were co-cultured for two days and then transferred to selection medium containing 100 mg/L kanamycin (Figure 26B). After 3 - 4 weeks of selection green shoots started appearing from the cut surface of the cotyledons (Figure 26C). The putative transgenic plants were sub-cultured on the same medium containing kanamycin (100 mg/L) to establish proper root system (Figure 27 A-D). The transgenic plants with well developed root system were transferred to soil and grown in the greenhouse for fruit setting (Figure 27E-F).

2. Tissue specific high-level expression

To achieve tissue specific high level expression for a transgene, our strategy was to express a modified T7 RNAP with a nuclear localization signal (NLS), to target the T7 RNAP to nucleus (Dunn *et al*., 1988), under a plant tissue specific gene promoter and express the transgene under T7 promoter and terminator in the same construct. To test this strategy, gene constructs containing differentially expressed gene promoters with various tissue specificities were used to and transform tobacco, tomato and rice. The pITB228, pITB450, pITB550, pITB650, pITB750 and pITB850 constructs contained *uid*A placed under T7 promoter and terminator sequences (Figure 29A) and the modified T7 RNAP with NLS was placed under the control of the small subunit of ribulose-bisphosphate carboxylase (*rbc*S-3A) (Kuhlemeier *et al*., 1988), stress inducible *kin1* (Wang and Cutler, 1995; Wang *et al*., 1996), *cor6.6* (Wang and Cutler, 1995; Wang *et al*. 1996), phenylalanine ammonia-lyase (*pal1*) (Ohl *et al*., 1990), and *pal1*Δ promoters (Ohl *et al*., 1990), promoter respectively (See construct of plasmids). For a direct comparison, the *uid*A was also placed directly under *rbc*S-3A, *kin1*, *cor6.6, pal1* and *pal1*Δ promoters, in pITB441, pITB541, pITb641, pITB741 and pITB841 constructs,

respectively. In addition, pCAMBIA1301 vector containing *uid*A under the control of a strong cauliflower mosaic virus (CaMV) 35S promoter (Benfy and Chua, 1990) that express constitutively in most tissue types was also transformed into tobacco and rice plants for comparison.

Tobacco transgenic plants were produced for each of the construct following *Agrobacterium* mediated transformation (Horsch *et al*., 1985). Southern hybridization (Figure 28) or polymerase chain reaction (PCR) was used to confirm the transformation. Northern blot techniques were used to confirm the transcription of *uid*A (Figure 29B, C). In Nt.450-2 plant, the *uid*A transcription under T7-system was 2 – 3 times higher when compared to *uid*A transcription directly under *rbc*S-3A promoter and the transcript levels were comparable to *uid*A transcripts under strong CaMV 35S promoter in Nt.1301-1 (Figure 29B).

As the transcript initiation from the T7 promoter by T7 RNAP was highly specific, primer extension analysis was carried out to authenticate the transcription of *uid*A by T7 RNAP. It can be seen from figure 29D, GUS transcripts initiated from the nucleotide 'G', specific for T7 promoter in Nt.450-2. These results clearly demonstrate that the T7 RNAP recognize its promoter in the randomly integrated plant genome and transcribed *uid*A accurately, akin to its transcription in *E. coli* (Imburgio *et al*., 2000).

The GUS activities are usually indicated in pmol MU/min/mg protein (Unit) (Jefferson *et al.*, 1987). However, data on the widely used CaMV 35S promoter activity, for instance, vary over a broad range. The CaMV 35S promoter mediated GUS activities were reported to be 113,000 Units (average of 10 plants, Benfey *et al*., 1989), 321 Units (one selected plant, Sanger *et al.*, 1990), 500 Units (highest expressing plant, Comai *et al*., 1990) and 130,000 Units (one selected plant, Keil *et al.*, 1989) and 820 to 13,000 Units (Weinmann *et al*., 1994). Thus, to discussing the GUS expression levels, we were used all data in our experiment. In other hand

we were using the fluorometric assay in transgenic plants at same time to comparison expression level between different constructs.

The histochemical analysis revealed that the GUS expression under *rbc*S:3A promoter was limited to green tissues with maximum activity localized in leaves followed by stem and absent in roots (Figure 30A). Most significantly, similar tissue specific expression pattern was observed among the transgenics that were transformed with pITB450 construct (Figure 30A). On the other hand, GUS under CaMV 35S promoter expressed in all tissues tested. It can be noted that the intensity of blue colour was more in the Nt.450-2 leaves when compared to the Nt.441-1 leaves indicating that the expression level could be high in the Nt.450-2 plants. Further analysis with the cross sectioned stem and root tissues (insets) shown that while GUS expressed in all cell types within the stem in the Nt.1301-1 and Nt.228-2 plants (Figure 30B), the activity was localized to cortex (green tissue within the stem) in both Nt. 441-1 and Nt. 450-2 plants and was absent in the cambium-ring, vascular tissue (xylem and phloem) and in the middle pith tissue (Figure 30C). Within the roots, as expected, GUS activity was present in Nt.1301-1 and Nt.228-2 but absent in Nt441-1 and Nt.450-2 plants.

Quantification of GUS activity in various tissues further confirmed that the GUS expression under *rbc*S-3A promoter was highly tissue specific with the highest activity in leaves and lowest in roots (Figure 31C). On the other hand, GUS under 35S promoter expressed in all the tissues investigated and the activity was 3 times high in leaves and 15 times high in roots when compared to GUS expressed under *rbc*S:3A promoter in the respective tissues. The pattern of GUS expression was similar in both Nt.441-1 and Nt.450-2 plants with the highest activity in leaf followed by stem and roots. Significantly, expression of GUS was 3 - 4 fold high under *rbc*S:T7-expression when compared to the *uid*A expressed directly under *rbc*S-3A promoter, a level comparable to GUS expressed under the strong viral CaMV 35S promoter (Figure 31C).

To further confirm the high level tissue specific GUS expression observed under *rbc*S-3A promoter using T7-system, four additional promoters (*kin1, cor6.6, pal1* and *pal*1Δ) from *Arabidopsis thaliana* that were shown to express at different levels in different tissues were examined. The *kin1* and *cor6.*6 genes express in stem, roots and in reproductive tissues at high level (Wang and Cutler, 1995; Wang *et al.*, 1996). Within the leaf, the expression was more prominent in guard cells. In the present study, similar expression pattern was observed in transgenic plants transformed with pITB641 (Figure 32D) and pITB541 constructs where GUS was expressed directly under *cor6.6* and *kin1* promoters respectively. And GUS expressed more prominently in the guard cells and under T7-system for *cor*6.6 promoter was higher when compared with GUS expression observed under *cor6.6* promoter directly (Figure 32 D-E).

Within the roots, the GUS expression varied considerably in different regions, with more activity localized in the root cap and was absent in the root elongation zone and in root hairs (Figure 32J). Similar expression pattern was observed under T7-system for both *kin1* (Figure 32K) and *cor6.6* promoters. As expected, the intensity of blue colour in root and root cap region was higher in Nt.550-2 when compared with GUS expression observed in Nt.541-1 (Figure 32J-K). On the other hand, expression of GUS under 35S promoter (pITB228) was uniformly high in all zones of root (Figure 32L).

Analysis of phenylalanine ammonia-lyase (*pal1*) gene promoter from *Arabidopsis* revealed that the *pal1* promoter is highly tissue specific with maximum activity in the vascular tissue of roots and leaves. In the present study, a full length (*pal1*, +1 to -832) and a truncated (*pal1*Δ, +1 to -540) promoters that have same tissue specificity but differ in their strength were used to test the GUS expression under T7-system. For comparison, *uid*A was also expressed directly under both *pal1* and *pal1*Δ promoters. Under *pal1* promoter, expression of GUS was high in vascular tissue of roots (Figure 32H) and leaves. Similar expression pattern was observed for GUS under *pal*1 promoter using T7-system (Figure 32I). Again, the GUS

activity was higher in Nt.750-2 when compared to Nt.741-1. Similar results were obtained for *pal1*Δ promoter. Strong GUS activity was observed in the vascular tissue of leaf (Figure 32F) and roots under T7-system. In contrast, the GUS expression under *rbc*S:3A promoter was restricted to green mesophyll cells and was absent in the vascular tissue (Figure 32G). This was expected for *rbc*S-3A promoter as the *rbc*S expression is linked to presence of chloroplasts where the vascular tissue is devoid of them.

A detailed quantification of GUS activity in the leaf tissue of ten randomly chosen transgenic plants revealed that the GUS activity was significantly higher under T7-system when compared the GUS expressed directly under any promoter investigated. On an average, there was 3 - 10 fold increase in the expression under T7-system (Figure 31A-B). A remarkable feature of T7-expression system in plants was found to be uniform levels of transgene expression among independently transformed plants, as opposed to large variations found under direct expression of plant promoters. It is possible that very low expression of T7 RNAP may be just sufficient to transcribe the transgene at maximum level. This feature will be particularly useful in plants such as legumes, cereals and tree species where it is most difficult to transform and regenerate large number of transgenic plants required to identify high-expressing plant(s).

Although an expression level of 20 – 30% of total soluble protein (tsp) has been achieved for chloroplast transformed *uid*A through T7 RNAP mediated transcription (McBride *et al*., 1994), this system has not been used for the expression of any recombinant protein useful in agriculture or in industry so far. One reason could be that similar or even higher level of expression can easily be achieved for foreign proteins in chloroplasts through the use of conventional chloroplast genetic engineering that utilize the plastid transcription and translation machinery (Daniell *et al*., 2001, De Cosa *et al*., 2001; Daniell and Dhingra, 2002; Maliga, 2002, 2003). The other reason could be that the uncontrolled high level expression through T7 system (under CaMV 35S promoter) might have a

deleterious affect on plants. In the present study, as compared to pCAMBIA1301 the frequency of transgenic plants obtained was reduced to 50% when transformed with pITB228 construct (Figure 33A). Similarly, there was 30% reduction in the frequency of transgenic plants are obtained using pITB550 and pITB650 constructs as compared to pITB541 and pITB641 constructs (Figure 33B), respectively. This might be presumably due to very high level expression of *uid*A achieved through T7 RNAP under strong and constitutive promoters that are deleterious to the differentiating somatic embryos. However, there were no significant differences in the regeneration frequency when transformed with any of the pITB450, pITB750, pITB850 and pITB441, pITb741, pITb841 constructs (Figure 33C-D) or plants that were transferred with pITB228/pBin-tetR or pBin-tetR/pITB228 (Figure 33A) (see inducible expression below).

Similarly, *GluB1* promoter (constructs pITB204, pITB241 and, pITB342) and *PDS* promoter (constructs pITB345, pITB345D and pITB348) were constructs for rice and tomato transformation, respectively for expression in seed (rice) and fruit (tomato).

To test the wider application of T7-expression system in plants, we have examined the expression of GUS, ferritin and provitamin A gene in rice, a monocot plant and a major source of staple food worldwide. Biolistic mediated transformation (Cao *et al*., 1990) was followed to introduce pCAMBIA1301, pITB228, pITB204, pITB241 and pITB342 constructs into rice genome. A large number of putative transgenic plants, regenerated on hygromycin selection, were screened for the expression of GUS. Similar to tobacco, GUS activity under CaMV 35S promoter using T7-system was higher in leaves and roots of transgenic plants (with pITB228) when compared with GUS expressed directly under CaMV 35S promoter (Figure 34D-E). While for transgenic plants (transferred with pITB342) were shown low expression of GUS gene in leaf (Figure 34F) and not found expression in roots (Figure 34H). Quantitative analysis of leaves also showed that the GUS expression was 3-5 times high in

plants that were independently transformed with pITB228 construct when compared to GUS expression in plants that were transformed with pCAMBIA1301 (Figure 35A), while in plants that were transferred with pITB342 construct were very low and only 5-10% when compared to GUS expression in plants that were transferred with pCAMBIA1301. Also, as opposed to large variations observed in the GUS expression among the independently transformed plants with pCAMBIA1301, variations were minimum among the pITB228 transformed plants, similar to the observations made in tobacco. Similarly, in root, GUS expression was higher in plants that transformed with pITB228 when compared with GUS expression in plants that transformed pITB342 respectively (Figure 35B). It shown for plants that transferred with seed promoter (*GluB1*) is expressed low in leaf and absent in root, while the CaMV 35S promoter GUS expression is uniform in whole plant. To verify that the high expression of GUS in Os.228-2 is due to increase in transcription under T7-system, Northern blot analysis was carried out to verify the transcript levels. When compared to Os.1301-1, a higher GUS expressing plant, the *uid*A transcripts were 3 times more in the Os.228-2 plant (Figure 35C), suggesting that the increased level of GUS expression could be due to increase in *uid*A transcription.

3. Inducible expression

Inducible expression is a powerful tool in basic as well as in applied research. To test the inducible expression of foreign genes under T7-system, a previously tested tetracycline inducible expression system in plants was used (Gatz *et al*., 1992). Foreign gene placed under a modified CaMV 35S promoter was shown to be expressed normally but repressed completely when the Tet repressor protein is co-expressed in the same plant (Gatz *et al*., 1992). The foreign gene can easily be de-repressed by treating the plants/tissues with low concentrations of tetracycline (Gatz *et al*., 1992). For this purpose, tobacco plants were first transformed with pBin-tetR to express Tet repressor protein constitutively under CaMV 35S promoter. The Nt.BintetR-1 plant that expressed high levels of *tet*R was re-

transformed with pITB228 (Figure 3). For comparison, Nt.228-1 plant obtained in the previous experiments was used.

Kinetics of de-repression was followed by taking leaf samples at defined time intervals from tetracycline treated (Tc-treated) and untreated plants and assayed them for the presence of *uid*A transcripts by Northern blot analysis (Figure 29C), for GUS activity (Figure 36A-B) and real time polymerase chain reaction (Figure 36C-D). In the Northern blot analysis, a single band corresponding to the expected size of *uid*A transcripts was detected only in the tetracycline treated sample (Figure 29C). Reprobing the same membrane simultaneously with *uid*A and T7 RNAP probes revealed the presence of both *uid*A and T7 RNAP genes in the Tc-treated leaf. No transcripts could be detected in the untreated sample. Real time PCR experiment also confirmed the tetracycline inducible expression of GUS in the Nt.228tetR-1 plant (Figure 36C).

The GUS activity was detected after 12 hours of Tc-treatment with maximum activity at 48 - 60 hours (Figure 36A). The non-Tc-treated leaves showed very low activity throughout the test period. In the Nt.228-1 plant, the GUS activity remained high with no significant differences between treated and un-treated samples. The GUS activity in the Nt.228-1 plant was comparable to the activity observed after 48 – 60 hours of Tc-treated Nt.228+tetR-1 plant (Figure 36B). No significant difference was observed between the Tc-treated and untreated samples from Nt.1301-1 plant. The co-expression of both T7 RNAP and *uid*A only after Tc-treatment coupled with the presence of GUS activity clearly demonstrate the highly regulated expression of GUS under T7-system.

Our results clearly demonstrate that T7 RNAP based transcription can be used for high level and tissue specific expression of foreign genes in higher plants and the transgene expression can be regulated through inducible mechanisms. These results may have profound impact on biotechnological application of transgenic plants in agriculture, industry and in functional genomic studies. In addition to, a

remarkable feature of T7-expression with inducible system in plants was found high efficiencies of regeneration for transgenic plants and it is useful for constitutive strong promoters used in plant transformation such as CaMV 35S promoter (Figure 33A).