CHAPTER 5
DISCUSSION

Plant transgenic technology is growing at a rapid pace as one of the key channels to meet growing food demand of burgeoning human population. This tool of genetic engineering of crop plants is referred to as second green revolution by father of green revolution, Nobel Laureate Professor Norman Borlaug as he expected it to handle food need of growing human population. The transgenic industry is also predicted to make an important mark in medicine (edible vaccine, drugs etc.), horticulture (in particular floriculture) and environment (in particular bioremediation). One of the key limitations of transgenic technology has been the need of ecologically viable selection marker systems. The present investigations were aimed to identify an ecologically viable and simple selection marker systems that can replace the prevailing most prominently used marker systems which are believed to have negative impact on human health and ecosystem. Brief account of key findings and goals achieved during the course of present investigations are highlighted and discussed taking into consideration the prevailing literature in the following paragraphs.

5.1 CHARACTERIZATION AND EXPRESSION OF THE SYNTHETIC \textit{merBps} GENE

A distinct variation in codon usage among different plants, animals and microorganisms is well established (Lightfield et al., 2011; Muyle et al., 2011). Variation in codon preferences in different organisms is considered to be a significant barrier to heterologous gene expression. Rarely employed codons in the target gene can lead to poor translation, decreased mRNA stability and premature termination of translation or even misincorporation of amino acids in polypeptide/protein during translation (Sorensen et al., 1989; Duret, 2000; Mukhopadhyay et al., 2007). Direct experimental evidence for a positive correlation between codon usage and gene expression has been reported by transforming plants with vectors expressing genes with modified codon (Perlak et al.,
Variation in the abundance of various tRNAs is correlated with codon usage, which determines translation efficiency (Dong et al., 1996).

In present investigations, the nucleotide sequence of 639 bp long merB gene from E. coli plasmid R831b was considered as base. Earlier, Bizily et al. (1999) created merBpe gene by adding flanking regions towards both 5’ and 3’ end of E. coli merB gene for its effective translation in Arabidopsis. However, it has been proven beyond doubt that the gene(s) of choice inserted within T-DNA of Agrobacterium tumefaciens are expressed in plants even otherwise (Narasimhulu et al., 1996; Hong et al., 1997). Subsequently, with an opinion that the GC rich genes show less expression compared to AT rich genes in plants, Jilin et al. (2002) structured the merBhe gene with lowered GC content by modifying the sequence of the merB gene with the help of long primers, sense (forward) primer of 138 nucleotides and antisense (reverse) primer with 112 nucleotides, for better expression in plants. During present investigations efficacy of synthetic merB gene, designated as the merBps gene, which was designed in accordance with the codon usage in higher plants, in particular Nicotiana tabacum was tested. The nucleotide sequence of the merBps gene was confirmed through custom services from two independent companies. The merBps gene differed from the native merB gene in possessing (i) 143 altered of a total of 214 altered codons and (ii) an additional ATG codon towards 5’ end. Overall, the merBps gene consisted of 214 codons (including termination codon) with ~67% codons distinctly different from the merB gene.

5.2 EXPRESSION OF ORGANOMERCURY LYASE

The expression of the recombinant protein is an important step towards elucidating role of many genes discovered through genome sequencing projects and also for validating gene targets. Recent progress in the fundamental understanding of transcription, translation and protein folding together with serendipitous discoveries and
the availability of improved genetic tools have made *E. coli* more valuable than ever for the expression of even complex eukaryotic proteins (Makrides, 1996; Baneyx, 1999).

For effective purification of the protein encoded by the gene of interest, pET vectors are often used, as six codons that encode for histidine residues get ligated towards the 3’ end of the gene of interest (Novagen, 2002-2003). Accordingly, the recombinant protein encoded by the gene under test would possess additional six histidine residues towards C-terminus end. This enables easy purification of the recombinant protein through Nickel-nitrilotriacetate (Ni-NTA)-agarose affinity protein purification kit from Qiagen (Germany) under non-denaturing conditions (Nilsson et al., 1997). As expected organomercury lyase tagged with histidine residues was retained by the nickel agarose matrix. Hexa(His)-tagged organomercury lyase bound to nickel agarose beads could be easily displaced using elution buffer, as the attachment and detachment/displacement of His-tag with nickel is pH-dependent. It is known that the Histidine residues of hexa(His)-tag protein bind to nickel at specific pH and can be displaced by lowering the pH. SDS Poly Acrylamide Gel Electrophoresis (PAGE) analysis confirmed that the protein fractions collected subsequent to detachment of His-tagged organomercury lyase showed the presence of single band having a molecular weight of ~23 kDa. Western analysis also showed the presence of a single band that reacted specifically with polyclonal antibodies (rabbit) raised against organomercury lyase, suggesting that only hexa(His)-tagged organomercury lyase was bound to the nickel column. Presence of single protein band in SDS gel and a single band on nylon membrane reacting with antibodies raised against organomercury lyase clearly confirmed that the recombinant organomercury lyase expressed in *E. coli* Rosetta harbouring pET-29a(+)merBps could be purified to electrophoretic homogeneity.

The recombinant protein gets His-tagged when pET29a(+) vector like any other pET vector is used for expressing foreign gene, i.e. gene of one’s choice. Just like any recombinant protein produced using pET vector, recombinant organomercury lyase produced will contain six histidine residues at C-terminus.
During present investigations the merBps gene was successfully cloned into expression vector pET29a(+). Distinct primers, with BamHI restriction site in the forward primer and HindIII restriction site in the reverse primer, were used to amplify only the merBps gene from pUC19merBps plasmid. The resultant amplicon of the merBps gene could be successfully cloned into an expression vector pET29a(+) to obtain recombinant pET29a(+)|merBps plasmid. Restriction analysis with BamHI and HindIII confirmed the presence of the merBps gene in pET29a(+)|merBps plasmid.

The pET29a(+)merBps plasmid could be successfully mobilized into BL21(DE3) and Rosetta strains of E. coli. Expression of the merBps gene in both the strains of E. coli was tested in presence and absence of IPTG. Although, small quantity of protein corresponding to organomercury lyase in saline phosphate (pH 7.4) buffer extractable protein fraction of cells of both strains of E. coli cells was detected even without IPTG induction, the quantity of protein was significantly higher in cells subjected to IPTG induction, suggesting the importance of IPTG induction for effective encoding of the merBps gene to yield organomercury lyase protein. Necessity of IPTG for expression in E. coli is well documented (Hansen et al., 1998). The expression of the merBps gene was superior in cells of Rosetta strain as compared to that of BL21(DE3) strain. These results suggested that Rosetta strain of E. coli is superior for expression of synthetic merBps gene compared to BL21(DE3) strain. The Rosetta host strains are BL21 derivatives designed to enhance the expression of the eukaryotic genes that contain codons rarely used in E. coli (Brinkmann et al., 1989; Seidel et al., 1992; Kane, 1995; Kurland and Gallant, 1996; Baca and Hol, 2000). The tRNA genes are driven by their native promoters (Novy, 2001). The presence of translated product organomercury lyase in the protein fraction was determined through immunoblot analysis by using anti-organomercury lyase antibodies raised in rabbit.
5.3 NICOTIANA TABACUM L. VAR. PETIT HAVANA (TOBACCO) TRANSFORMATION

In order to evaluate effectiveness of the merBps gene, model plant Nicotiana tabacum L. var. Petit Havana was used. For expression in higher plants, it is important to clone the candidate/foreign genes in desired binary plant expression vector (Komori et al., 2007). For the present investigations the merBps gene was cloned in the plant expression vector pBinAR. pBinAR binary vector used for plant transformation had kanamycin resistance gene (nptII) and multicloning sites between CaMV35S promoter and ocs terminator (Hofgen and Willmitzer, 1990). The CaMV 35S promoter is a strong, constitutive promoter used in many transformation studies (Benfey and Chua, 1990). The merBps gene was successfully cloned in the MCS region of pBinAR in such a way that it would be under the control of CaMV 35S promoter with the octapine synthase (ocs) terminator. This resultant vector referred to as pBin-merBps besides, having the merBps gene also contained the nptII gene under the control of nos promoter and nos terminator. The presence of both merBps and nptII genes in this recombinant vector was confirmed through PCR analysis by using respective gene specific primers. The pBin-merBps vector was mobilized into Agrobacterium tumefaciens strain EHA105. The resultant Agrobacterium tumefaciens strain EHA105 harbouring the pBin-merBps was used for transformation of Nicotiana tabacum L. var. Petit Havana (tobacco).

Leaf segments are recognized as ideal explants for regeneration and transformation of tobacco (Horsch et al., 1985). Even during present investigations rapid callogenesis and caulogenesis (both direct and through callus) was recorded from the leaf segments on MS medium supplemented with 2 mg/l BAP and 0.1 mg/l NAA. A significant reduction in callogenetic and caulogenetic potential of leaf segments that were co-cultivated with A. tumefacienciens strain EHA105 harbouring pBin-merBps on MS medium supplemented with 2 mg/l BAP and 0.1 mg/l NAA in presence of 500 mg/l cefotaxime was observed.
“Efficacy of synthetic merB gene as selection marker”

Only a minute fraction of hundreds of cells associated with explants co-cultivated with Agrobacterium are transformed and it is necessary to distinguish transformed cells from non-transformed cells in order to specifically multiply and obtain regeneration/morphogenic units only from the transformed cells. This necessitated the scientific world to look for a suitable device to perform this important task, which lead to the emergence of selection marker system. Emergence of selection system, consisting of a marker gene and a selection agent, as appropriate device to distinguish limited transformed cells from a massive number of untransformed cells dated back to 1983 when two independent research groups (Fraley et al., 1983 and Bevan et al., 1983) found the nptII gene as a marker gene to distinguish/identify transformed/transgenic cells with kanamycin and other glycocidic antibiotics as selection agents. In general, all the positive conditional marker genes, which constitute predominant marker genes that are widely used for plant transformation technology, have potential to detoxify certain molecules/metabolites that are known to kill non-transformed plant cells (Bevan et al., 1983; Fraley et al., 1983; Waldron et al., 1985; DeBlock et al., 1989).

The selection pressure is one of the most important factors affecting transformation efficiency because only a limited number of cells are transformed during transformation (Haldrup et al., 1998). If the selection pressure is too low, more escapes will be regenerated due to low concentration of selection agent. On the contrary, if the selection pressure is too high, even the transformed cells will not grow or even die either because of inhibitors secreting from dying untransformed cells or because of disturbance in the transport of essential nutrients through dying tissues to the living transformed cells (Haldrup et al., 1998). Further, significant amount of selection agent may not reach the encoded marker gene product i.e. the concerned enzyme and instead randomly reach sites where essential cellular metabolism might be going on. It is also likely that the quantity of marker gene encoded protein/enzyme may not be sufficient to detoxify the significant proportion of the selection agent. Moreover, different plant systems based on the taxonomic unit (such as monocots and dicots; families, genera, species, varieties/lines,
genotypes) exhibit different degrees of resistance/tolerance to different selection agents (Miki and McHugh, 2004; Zang et al., 2005).

5.4 SUITABILITY OF KANAMYCIN AS A SELECTION AGENT

The plant expression vector pBinAR, used during present investigations possesses the *nptII* gene as the selection marker gene (Hofgen and Willmitzer, 1990). Accordingly, more commonly used glycosidic antibiotic, kanamycin, was used as selection agent. However, the effective concentration of kanamycin to kill cells vary amongst plants from different taxonomic groups and may also vary between varieties of species of certain plant systems (Miki and McHugh, 2004; Zang et al., 2005). For instance, monocots are often insensitive to relatively high levels of kanamycin, which allows regeneration of untransformed plant cells on kanamycin-containing medium. For example concentration of 100 mg/l kanamycin allowed growth of 70% of untransformed rice calli (Dekeyser et al., 1989), adventitious shoot formation on flower stem explants of tulip was noted in the presence of 500 mg/l kanamycin (Wilmink and Dons, 1993) and protoplasts of *Lolium perenne* were able to divide even in the presence of 800 mg/l kanamycin (Potrykus et al., 1985).

In general, kanamycin is used as selection agent at concentration ranging between 50-300 mg/l i.e. 86-516 µM depending on plant species. During present investigations callogenesis, caulogenesis and overall regeneration potential of leaf segments on MS medium supplemented with 86 µM (50 mg/l) to 129 µM (75 mg/l) did not differ significantly from that grown in absence of kanamycin. Therefore, for subsequent experiments 172-344 µM (100-200 mg/l) of kanamycin was used for distinguishing transformed cell lines from non-transformed cell lines. However, the degree of expansion and callusing of co-cultivated leaf segments of *Nicotiana tabacum* L. var. Petit Havana decreased with increase in the concentration of kanamycin above 129 µM (75 mg/l). Number of shoots obtained per explant decreased with increase in the concentration of kanamycin from 172 to 344 µM (100 to 200 mg/l). The number of shoots obtained per
leaf segment in presence of 100 mg/l (172 µM), 150 mg/l (258 µM) and 200 mg/l (344 µM) kanamycin selection regimes were ~8.4, 7.2 and 5.0, respectively. The shoots obtained from different leaf segments and different parts of each leaf segment were taken into consideration as independent putative transformants.

Only ~6, 12 and 45 percent of shoots of independent putative transgenic lines selected in selection regime of 172, 258 and 344 µM kanamycin showed PCR amplification with nptII specific primers, indicating the presence of the nptII gene only in these putative transgenic lines. In other words, a large number of escapes were obtained even in presence of kanamycin. This may be accounted to either the lack of kanamycin to effectively reach/penetrate into these cells (which could be due to presence of a number of layers of dead cells/tissues beneath/around them) or due to certain somaclonal variations that could have enhanced tolerance of untransformed cells to kanamycin (Philips et al., 1994). It is also likely that the expression of the nptII gene might not be appropriate in few transformed cell lines. The shoots that were obtained from the cells that do not have any means to deactivate/detoxify kanamycin and are not transformed generally turn yellow and show necrosis during subculturing. Only about 1.2% of the explants possessed the cells with potency to produce shoots i.e. exhibit caulogenesis in the presence of kanamycin. A number of earlier researchers also often recorded a large numbers of escapes i.e. non-transformed cell lines with relatively good degree of resistance to kanamycin (Mentewab and Stewart, 2005).

As over 50% of the putative transgenic lines selected even at a selection regime of 344 µM kanamycin were found to be non-transgenic, kanamycin can not be considered as a stringent selection agent for effectively distinguishing true transformed cell lines from non-transformed cell-lines in case of Nicotiana tabacum L. var. Petit Havana. Four transgenic lines of Nicotiana tabacum L. var. Petit Havana designated as TK1, TK2, TK3 and TK4 were selected based on vigorous shoot growth and overall strength of shoot system on MS medium containing 344 µM (200 mg/l) kanamycin.
5.5 SUITABILITY OF PMA AS A SELECTION AGENT

The plant expression vector pBin-\textit{merBps}, used during present investigations also possessed the \textit{merBps} gene under the control CaMV 35S promoter and the octapine synthase terminator. Accordingly phenyl mercury acetate (PMA) was used as selection agent. Organomercurial compounds such as methyl mercury chloride or PMA can retard or kill plants when present at concentration of 0.1 µM and above (Bizily et al., 1999, Jilin et al., 2002).

During present investigations, 1 µM of PMA was used for identifying/distinguishing transformed cell lines from non-transformed cell lines. No callogenesis and/or caulogenesis was observed from leaf segments on MS medium supplemented with PMA at levels above 1.5 µM. Further, even in presence of 1 µM PMA the degree of expansion and callusing in co-cultivated leaf segments of \textit{Nicotiana tabacum} L. var. Petit Havana was significantly low compared to that noted in presence of 344 µM (200 mg/l) kanamycin. Only about 21% of the explants coped to produce cells with potency to exhibit caulogenesis i.e. to produce shoots in the presence of 1 µM PMA. Number of shoots obtained per explant also decreased remarkably. In fact, number of shoots obtained per leaf segment with 1 µM PMA selection regime was only ~0.21. Shoots obtained from different leaf segments and different parts of each leaf segment were taken into consideration as independent putative transformants.

Interestingly, during present investigations, 100 percent of shoots of independent putative transgenic lines of tobacco selected in presence of 1 µM PMA as selection agent, showed PCR amplification with the \textit{merBps} gene specific primers, indicating the presence of the \textit{merBps} gene in all these putative transgenic lines and absolutely no escapes i.e. no non-transformed cell lines were obtained in presence of 1 µM PMA selection regime. Non transformed leaf segment explants were totally bleached (turned white), while the transformed explants were able to produce the cells with a potency to produce direct shoots. Another four transgenic lines of \textit{Nicotiana tabacum} L. var. Petit Havana
designated as TP1, TP2, TP3 and TP4 were selected based on vigorous shoot growth and overall strength of shoot system on MS medium containing 1 µM PMA.

These results, accomplished during present investigations, proves beyond doubt that the selection system consisting of the merBps gene as marker gene with PMA as selection agent is far superior to the selection system consisting of the nptII gene as marker gene with kanamycin as selection agent for distinguishing/selecting the transgenic cell lines from non-transgenic cell lines in *Nicotiana tabacum* L. var. Petit Havana.

Earlier, Choi et al. (2005) claimed that the merB gene from *Streptococcus aureus* can be used as an excellent plant selectable marker. These researchers claimed to had obtained callus induction from 70-80% of co-cultivated stem explants in presence of 5 µM methyl mercury. Without furnishing any valid data, these investigators also claimed shoot regeneration from all the calli in presence of 5 µM methyl mercury.

5.6 MOLECULAR ANALYSIS OF TRANSFORMANTS SELECTED WITH PMA AND KANAMYCIN

Polymerase chain reaction analysis is the basic and powerful tool used for rapid screening of transformants from non transformed escapes (Edwards et al., 1991; Wang et al., 1993). Even during present investigations PCR analysis was carried out for initial screening of putative transformants selected using PMA or kanamycin as selection agents. DNA was isolated from the shoots of both sets of the putative transformants was subjected to PCR analysis with nptII gene specific primers as well as merBps gene specific primers. The DNA from all the merBps transgenic lines yielded 587 bp long amplicon with the merBps gene specific primers and 780 bp long amplicon with the nptII gene specific primers, suggesting the presence of both nptII and merBps genes.

These results clearly indicated the presence i.e successful integration of complete T-DNA harbouring the merBps gene along with nptII in selected putative transgenic lines. It further confirmed the functionality of the merBps gene as selection marker gene
for the selection of transformants from non-transformants is perfect and proves beyond doubt that PMA is a powerful stringent selection agent for selecting transgenic lines from non-transgenic lines in *Nicotiana tabacum* L. var. Petit Havana over frequently used antibiotic, kanamycin. Based on overall vigor eight of the PCR positive independent transgenic lines showing amplification with both *merBps* and *nptII* genes were chosen for further characterization.

The PCR analysis of putative transformants maintained in vitro is often not considered to be reliable as possibility of *Agrobacterium* remaining in association with putative transformants cannot be ruled out (Veluthambi et al., 2003). Therefore, the PCR analysis was repeated with DNA isolated from the leaves of three month old independent putative transformants/transgenic lines grown in green house in pots. However, there are reports stating the likely presence of *Agrobacterium* association even in field grown plants until the end of its life cycle (Yin and Wang, 2000). Therefore it was necessary to further confirm the integration of *merBps* gene in the genome of tobacco through southern analysis.

### 5.6.1 Southern Analysis

PCR analysis cannot firmly be taken as a surety for the successful integration of the gene of one’s choice (such as the *merBps* gene used during present investigations) as the integration of T-DNA could be only partial (Pniewski and Kapusta, 2005). In addition, although PCR amplification is a good indicator for integration of the gene(s) in a large number of instances, it cannot certainly provide any information regarding number of copies of T-DNA/transgene that got integrated in each of the transformants. Southern analysis is an ideal means to determine the number of loci in the plant genome where the transgene got integrated, which is often equated to the number of copies of the transgene in the transgenic line (Prasad et al., 2000). The *Eco*RI digested genomic DNA, from leaves of wild type and the *merBps* transgenic lines, resolved on agarose gel and probed with (*α*-32P) dCTP *merBps* gene confirmed the successful integration of *merBps*
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gene in all the eight transgenic lines tested. As is evident from these experiments seven of these transgenic lines possessed single copy, while one possessed two copies of the merB gene. The genotypes having single copy of gene are often considered superior to the ones having two or more copies of integration as latter often lead to silencing of the transgene in subsequent generations (Matzke and Matzke, 1991; Stam et al., 1997; Matzke et al., 2000; Waterhouse et al., 2001; Veluthambi et al., 2003; Wang et al., 2003). The transgenic lines showing the presence of single insert of the merB gene were advanced and self pollinated with an aim to get homozygous lines.

5.6.2 Effective Transcription of The merB Gene

Subsequent to the confirmation of the presence of merB gene in the merB transgenic lines of Nicotiana tabacum L. var. Petit Havana selected using PMA or kanamycin as selection agents, through PCR and Southern blot analysis, the effective transcription of this transgene was tested. In order to authenticate effective transcription of synthetic merB gene in the selected merB transgenic lines (with PMA or kanamycin as selection agents), total RNA isolated from selected merB transgenic lines was subjected to Northern, RT-PCR and real-time (qRT) PCR analysis. The merB gene labeled with (α-32P) dCTP was used as probe.

The Northern blot technique is established to study gene expression using RNA (Kevil et al., 1997). In the first set of experiments, resolved total RNA transferred on to nylon membrane was probed with radiolabelled (α-32P dCTP) full length merB gene. Only a single RNA band in each lane of total resolved RNA of eight independent transgenic lines showed radiolabelling due to hybridization of the mRNA with radiolabelled merB probe, establishing that merB gene in all the eight selected merB transgenic lines of Nicotiana tabacum L. var. Petit Havana had been effectively transcribed. However, the intensity of the band varied between the transgenic lines portraying significant variation in transcription capacity amongst them. Transgenic lines TP2, TK1 and TK3 showed higher intense band indicating their higher potential to
express the *merBps* gene as compared to other transgenic lines. As anticipated none of the resolved RNA bands of wild type hybridized with radiolabelled *merBps* probe, revealing the absence of any RNA that resemble the *merBps* transcript.

In the second set of experiments, semi-quantitative RT-PCR was performed to analyze the level of mRNA transcript in the leaves of the *merBps* transgenic lines of *Nicotiana tabacum* L. var. Petit Havana. For Reverse transcription polymerase chain reaction (RT-PCR), RNA isolated from selected *merBps* transgenic lines was first reverse transcribed into its DNA complement (complementary DNA, or cDNA) using the enzyme reverse transcriptase, and the resulting cDNA was also further, used for carrying out traditional PCR and real-time PCR.

All the eight *merBps* transgenic lines displayed the expected 587 bp amplified fragment in RT-PCR, demonstrating active transcription of the *merBps* gene in them. As expected, RNA isolated from leaves of wildtype *Nicotiana tabacum* L. var. Petit Havana failed to show any RT-PCR amplification with the *merBps* gene specific primers. In contrast, the RT-PCR with actin gene transcript, which was used as an internal control, showed the synthesis of ~60 bp amplicon with actin gene specific primers equally well in wild type and in all the eight *merBps* transgenic lines. None of the RNA samples showed amplification with *Taq* DNA polymerase, ruling out any possibility of transcripts in the RNA samples contaminated with any DNA.

Third set of experiments involved comparative analysis of the *merBps* gene expression in the selected *merBps* transgenic *Nicotiana tabacum* L. var. Petit Havana lines through Real-Time PCR.

Real time –PCR has been proven to be simple, fast and effective/powerful tool for high throughput quantitative analysis of various genes including transgene(s) in living systems (Bako et al., 2011). Real time PCR enables to quantify absolute and relative gene expression. Real time –PCR is an authentic tool to confirm the integration of fully functional transgene (i.e. it ensures not only integration of full length transgene but also
determines its effective transcription) in the transgenic lines that are selected using any means of selection (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Real time –PCR is a powerful tool for exploring RNA transcript levels of transgenes in transgenic plants (Bako et al., 2011). L25 ribosomal protein which is one of commonly used internal reference gene was used for data normalization (Schmidt and Delay, 2010).

For comparative analysis of merBps gene expression in transgenic tobacco plants, Real-Time PCR was carried out. \( \Delta C_T \) values indicate the expression of gene of interest relative to internal control. During present investigations, we calculated \( \Delta C_T \) value of merBps gene in transgenic lines using L25 ribosomal protein gene as internal control. \( \Delta C_T \) values varied significantly amongst different independent merBps transgenic tobacco genotypes and ranged between -0.21 to -0.486.

Real time PCR analysis, in addition to Northern blot and RT-PCR analysis, authentically confirmed effective transcription of the merBps gene in all the eight merBps transgenic lines, irrespective of the selection agent (PMA or kanamycin) that was used for selecting these transgenic lines.

**5.6.3 Effective Translation of The merBps Gene**

Immunoblot (Western blot) analysis of protein bands, of crude enzyme extract in PBS buffer from the leaves of transgenic lines of Nicotiana tabacum selected from PMA or kanamycin as selection agents resolved on polyacrylamide gel and transferred on to nylon membrane, showed the presence of a ~23 kDa protein band that cross reacted with polyclonal antibodies (rabbit) raised against organomercurial lyase (purified from E. coli Rosetta harbouring pET-29a (+) containing merBps gene). In contrast, no bands of the extract from wild-type plants showed any cross reaction with the antibodies indicating absence of any protein similar/corresponding to organomercury lyase. These results convincingly demonstrated effective translation of merBps gene to produce ~23kDa polypeptide/protein in all the eight transgenic lines tested.
5.7 DETERMINING THE STRENGTH OF THE merBps TRANSGENIC LINES TO WITHSTAND PMA

It was evidently apparent from the molecular analysis that the synthetic merBps gene got effectively (i) integrated in genomic DNA; (ii) transcribed; and (iii) translated. In order to evaluate if the merBps encoded organomercury lyase is functional in the selected merBps transgenic lines of Nicotiana tabacum L. var. Petit Havana, it was necessary to establish the potential of these transgenic lines to withstand/detoxify organomercurials. As phenyl mercury acetate is one of most common, easily available and cheaper amongst organomercurials, during present investigations effectiveness of the selected merBps transgenic lines to withstand and detoxify different levels of PMA was determined. Five independent sets of experiments were carried out to establish the effective functioning of the merBps encoded organomercury lyase by determining the potential of these transgenic lines to withstand extremely toxic organomercurials.

In the first set of experiment, performance of leaf segments of wild type and selected merBps transgenic lines of Nicotiana tabacum L. var. Petit Havana were cultured in MS medium supplemented with BAP (2 mg/l) and NAA (0.1 mg/l) in presence of different levels (1-4 µM) of PMA. All the leaf segments of wild type bleached/chlorotic and turned white within seven days in presence of PMA even at concentrations as low as 1 µM, without exhibiting any signs of growth. In contrast, leaf segments of all the merBps transgenic lines of Nicotiana tabacum L. var. Petit Havana tested not only remained green but also showed callogenesis as well as caulogenesis in all the three concentrations used i.e. 1 µM, 2 µM and 4 µM. These results show the merBps transgenics of Nicotiana tabacum L. var. Petit Havana irrespective of whether selected using PMA or kanamycin as selection agent, possessed potential to effectively detoxify toxic PMA (organomercurial). These results conclusively prove that the organomercury lyase encoded/produced in the merBps transgenic lines is fully functional. Choi et al. (2005) failed to record callogenesis from stem segments of wild type hybrid poplar in presence of methyl mercury. However, the merB transgenic plants grew well even at
concentrations as high as 5 µM. Based on the tolerance of the merB transgenic plants of hybrid poplar to methyl mercury, Choi et al. (2005) claimed that the merB gene from Streptococcus aureus can be used as an excellent plant selectable marker.

Earlier, Bizily and co-workers recorded strong inhibitory effect of organomercurial compounds and seed germination and early seedling growth in wild type Arabidopsis thaliana. Therefore, in the second set of experiment seed germination and early seedling growth of both wild type and the merBps transgenic lines of Nicotiana tabacum L. var. Petit Havana was evaluated in ½ MS medium supplemented with 2 mg/l BAP and 0.1 mg/l NAA along with 1 µM PMA. Although seeds of both wild type and the merBps germinated and exhibited early seedling growth equally well in absence of PMA, but in presence of 1 µM PMA seeds of wild type failed to germinate. On the contrary, the seeds of all the merBps transgenic lines of Nicotiana tabacum L. var. Petit Havana irrespective of whether they were selected using 1 µM PMA (i.e. TP2 and TP4) or 344 µM (200 mg/l) kanamycin (i.e. TK1) as selection agent, germinated and exhibited early seedling growth. These results also convincingly prove that the merBps gene encoded organomercury lyase is fully functional and hence has potential to effectively detoxify highly toxic PMA. Introduction of merBpe gene, reconstructed by Bizily et al. (1999) with an upstream sequence typical of plant genes, enabled transgenic Arabidopsis thaliana to tolerate organomercurials and allowed its seed to germinate and seedlings to grow at concentration of 0.1 to 2 µM.

In the third set of experiments potential of nodal axillary buds of both wild type and the merBps transgenic lines of Nicotiana tabacum L. var. Petit Havana to sprout and form shoots in MS medium supplemented with BAP (2 mg/l) and NAA (0.1 mg/l) was evaluated in presence of different levels of PMA. In general, axillary buds of nodal explants of both wild type and merBps transgenic lines sprouted rapidly and formed shoots in MS medium not supplemented with PMA. However, in presence of PMA even at a concentration of 1 µM nodal explants of wild type rapidly got decolorized resulting in necrosis. On the contrary, nodal explants of all the tested merBps transgenic lines of
Nicotiana tabacum irrespective of whether selected with PMA or kanamycin as selection agent showed axillary buds sprouting resulting in the emergence of shoots in presence of PMA. However, the growth of shoots in all the merBps transgenic lines were significantly curtailed with increase in concentration of PMA. These set of investigations also further supported the fact that the merBps gene encoded organomercury lyase is fully functional and possesses potential to detoxify PMA.

In the fourth set of experiments, potential of 2-3 cm long healthy shoots of both wild type and the merBps transgenic lines of Nicotiana tabacum to withstand different levels of PMA was tested. As anticipated, both wild type and the merBps transgenic lines grew equally well in absence of PMA, but in presence of 1 µM PMA the shoots of wild type failed to show any growth. Rather, the shoots of wild type showed rapid loss in green pigmentation and drying to begin with from tip of the leaves spreading to the rest of each leaf. Often, several leaves of wild type turned completely white in presence of PMA. In contrast, shoots of all the merBps transgenic lines retained green pigmentation and grew. However, the growth of shoots of the merBps transgenic line was significantly curtailed with increase in concentration of PMA.

Earlier, PMA had been shown to inhibit Hill reaction, photophosphorylation (Siegenlthaler & Packer, 1965), the dark reduction of cytochrome C554 and ferredoxin or ferredoxin-NADP oxidoreductase (Hiyama et al., 1970). Inhibition of PS II by PMA appears to be selective, blocking a site between the 3-(3,4-dichlorophenyl)-1,1-dimethyl urea-sensitive site and the site inactivated by high concentration of tris buffer (Honeyeutt & Krogmann, 1972).

In the fifth set of experiments, response of leaf discs from healthy leaves of three month old plants of wild type and the merBps transgenic lines of Nicotiana tabacum floated in distilled water with 0, 1, 2 and 4 µM PMA was evaluated. Amazingly, all the leaf discs of wild type lost green pigment and turned white within six days in presence of PMA even at concentration as low as 1 µM. In contrast, leaf discs of both the transgenic
lines tested remained green even after 20 days. Even though, the leaf discs of both the transgenic lines remained green, the level of Chl a declined partially in one line and significantly in another one with increase in concentration of PMA. It is interesting to note that the leaf discs of wild type although turned visibly white upon exposure to PMA, only about 25% loss in Chl a was recorded upon exposure to 4 µM PMA, which infact was closer to the decline in Chl a content recorded in one of the merB transgenic line. In contrast the Chl a content of another merB transgenic line remain almost unaltered upon exposure to PMA. However, a steep decrease in the levels of Chl b was recorded in the leaf discs of wild type exposed to even 1 µM PMA. It is known that organomercurial compounds strongly inhibit chloroplast functions, including electron transport, oxygen evolution (Bernier et al., 1993), Hill reaction, photophosphorylation, chlorophyll fluorescence (Kuper et al., 1996) and chlorophyll content (Sen and Mandal, 1987).

It is interesting to note that the carotenoid level in the leaf discs exposed to even 1 µM PMA dropped to zero in case of wild type. The leaf discs of both the transgenic lines showed about 20 to 30% decline in carotenoid level upon exposure to 4 µM PMA. Inspite of sincere/best efforts, no published report on impact of PMA on carotenoid content is available. It would be of interest to elaborate further on impact of organomercurials on carotenoid content in plants.

It is also equally obvious from the fifth set of experiments that the organomercury lyase encoded by the merB gene is fully functional as leaf discs of both the merB transgenic lines possessed potential to tolerate toxic levels of PMA.

It is clear from above five independent sets of investigations that the synthetic merB gene which encodes organomercury lyase has been successfully integrated, transcribed, translated and is fully physiologically functional in all the independent merB transgenic lines of Nicotiana tabacum L. var. Petit Havana. It is evident that the
merBps gene encoded organomercury lyase must be detoxifying PMA through following reaction:

\[
\text{Organo} + \text{organomercury lyase} \rightarrow \text{organo} + \text{Hg}^{2+} + \text{Cl}^{-} + \text{OH}^{-}
\]

5.8 ADVANTAGES OF merBps AS SELECTION MARKER WITH PHENYL MERCURY ACETATE (PMA) AS STRINGENT SELECTION AGENT

Firstly, to the best of our knowledge the merBps gene with a size of 639 bp is one of the smaller marker genes known. Secondly, it has been designed in accordance with codon usage for its optimal expression in higher plants especially tobacco. Thirdly, unlike majority of alternate genes such as DHFR (for dihydrofolate reductase) (Herrear-Estrella et al., 1983; Eichholtz et al., 1987; Irdani et al., 1998), AK (for aspartate kinase) (Tewari-Singh et al., 2004), ocs (for octopine synthase) (Koziel et al., 1984) etc. that have been proposed to be used as an alternate to antibiotic/herbicide resistant genes, could have negative impact on general metabolism of amino acids such as lysine, threonine, methionine, cysteine and histidine and bases/nucleotides such adenine, thymidylic acid, merBps gene to the best of our knowledge would not have any negative impact on cellular metabolism. Fourthly and most importantly, use of PMA as a selection agent would be most economically viable as 1 g of PMA costs ~US$ 1.4 in comparison to kanamycin, hygromycin, glycosphate, phosphinotinic, methionine sulfoximine, methotrexate, L-lycine, L-threonine, mannose, and xylose which cost US$ 25.6, 472.5, 21.7, 441, 1536, 726, 33, 12.6, 1.6 and 1.9, respectively. Further, the effective concentration of PMA for selection is 1 µM (i.e. ~0.34 mg/l) which is several times less than effective concentration of several other selection agents in particular mannose, xylose, kanamycin, glycosphate, phosphothricin and L-lysine which are used in the range of 2-20 g/l (11.1-111.1 mM), 3-20 g/l (19.98-133.2 mM), 0.050-0.500 g/l (85-858 µM), 33-84 mg/l (2-5 mM), 20 mg/l (100 µM) and 0.292 g/l (2 mM), respectively. Effective
cost of using the *merBps*-PMA selection system is compared with major selection systems used for raising transgenic genotypes (Table 5.1). In addition *merBps* gene can be used across the taxonomic groups unlike several marker genes which have restricted utility. In addition, use of the *merBps* gene may not have any negative impact on health of human being or ecosystem/environment as this gene is widely distributed in microbial kingdom. It should be kept in mind the selection agent would be used only for identifying the transformed from the non-transformed. Therefore, use of organomercury compound as selection agent should not be taken otherwise.