Results
Different expression systems are being tried to express malaria vaccine candidate antigens (Lalitha et al., 1999; Hui et al., 1994; Burghaus et al., 1999). Plant has successfully been shown as a good expression system to produce a number of foreign proteins (Domansky et al., 1995; Liu et al., 1994; McGravey et al., 1995) either to use them as edible vaccine system (Arntzen, 1997) or to produce them in large scale. We, therefore, decided to express the MSP1$_{19}$, a C-terminal end of a leading malaria vaccine candidate antigen Merozoite Surface Antigen-1 (MSP-1) from P.falciparum, in plants.

Expression of the msp1$_{19}$ gene in plant involves different strategies and steps like cloning, transformation (bacterial and plant) and regeneration processes. To initiate our study, we have cloned msp1$_{19}$ gene in tobacco plant. In this chapter, results regarding cloning, transformation, purification and characterization of the plant derived MSP1$_{19}$ protein have been described.

To study the expression of msp1$_{19}$ gene in plant system, we initiated our study by expressing this antigen in tobacco plant and this chapter describes the expression and results obtained during the course of our study. Efforts were made basically keeping in mind the standard problem in plant transformation i.e., the low expression level of foreign protein in plants. Two different vector systems were tried in the present study to obtain high level of expression of MSP1$_{19}$.

**Expression of MSP1$_{19}$ protein in bacterial system**

Recently, Lalitha et al. (1999) have successfully expressed the msp1$_{19}$ gene in E. coli in soluble form and have characterized the bacterially expressed MSP1$_{19}$ protein. Briefly, the gene coding for MSP1$_{19}$ protein was PCR amplified from an Indian isolate routinely being maintained in vitro culture at ICGEB. The gene was then cloned into pQE expression vector, which added His-tag (Histidine-tag) at the N-terminal end of the protein. MSP1$_{19}$ protein was expressed in soluble form by inducing with IPTG. Fig 8 shows the
was expressed in soluble form by inducing with IPTG. Fig 8 shows the expression and purification of the reduced and non reduced *E. coli* expressed MSP1<sub>19</sub> protein. The purified protein reacted very well with most of the conformation as well sequence specific antibodies provided by Dr. Tony Holder (2F.10, 111.4, 1E1, 8a-12) and G.17.12 provided by Dr. S. Longacre.

**A. VECTOR SYSTEM I**

**Construction of vector for plant transformation**

At the beginning of our study, a commonly used vector pBI 121 (Clontech, USA) was tried to express transformed tobacco plants and to express MSP1<sub>19</sub> protein.

**Cloning of msp1<sub>19</sub> gene in vector pBI121**

To start with, gene corresponding to MSP1<sub>19</sub> was amplified using following primers.

Forward primer: 5' CGC GGA TCC ATG TTA AAC ATT TCA CAA CAC CAA TGC 3' and

Reverse primer: 5' CAA GAG CTA AGT TAG AGG AAC TGC AGA A A 3' (life technology, USA)

The forward primer has a BamHI site at 5' end, where as the reverse primer has a Sacl site.

PCR amplified fragment was cloned into pBI121. PBI121 vector (Clonetech) is a well established plant transformation vector, which has one 'right' border (RB) and a 'left' border (LB) as well as a marker gene (ie., nptll, kanamycin resistant) and reporter gene (gus) in the T-DNA. msp1<sub>19</sub> fragment was PCR amplified and double digested with BamHI/Sacl enzymes, and subsequently cloned in BamHI/Sacl digested pBI121. Thus, gus gene was removed from pBI121 (by BamHI/Sacl digestion) and the msp1<sub>19</sub> fragment replaced the gus
Fig. 8  A. Recombinant *E. Coli* protein profile. -: uninduced protein profile; +: IPTG induced protein profile.

B. Ni column purified 19 kDa recombinant MSP1$_{19}$ from *E. Coli*, R: Reduced protein; NR: Non-reduced protein
Results

gene under the direct control of 35S CaMV promoter (Fig 9). Newly constructed vector was termed as pPM121, which had two sets of complete genes, 1) kanamycin resistant gene (nptII) under nos-promoter and 2) msp1₁₉ gene under the control of 35S CaMV promoter along with a nos terminator, within the T-DNA. Thus, the chimeric construct was ready for the transformation.

Preparation of Agrobacterium transformation vector

Chimeric vector pPM121 was introduced in the disarmed Agrobacterium strain LBA4404. Positive clones were thoroughly screened in kanamycin (50mg/L) as well as streptomycin (50mg/L) plates. Directly clones were picked up from the plates and with the above mentioned primers colony PCR were performed to check the presence of msp1₁₉ gene. Positive clones (termed as pPMS clones) were selected and later on used for the plant transformation (fig 9).

Plant transformation and regeneration

Before transformation was attempted tobacco (tissue culture grown) leaves were cut into pieces and put in MS media with varying amount of NAA and BAP. Among the 0.5ppm BAP and 0.1ppm NAA, 0.5ppm BAP and 0.5ppm BAP, 1ppm BAP and 0.1ppm NAA, 1 ppm BAP and 0.5ppm NAA, 2ppm BAP and 0.2ppm NAA combinations. Best result (fast and healthy callus production as well as shoot initiation) was achieved in 1ppm BAP and 0.1ppm NAA (Table5). Therefore, that particular combination was selected for further studies. In subsequent experiments to achieve a suitable kanamycin dose varying amounts of kanamycin like, 10, 20, 30, 40 and 100mg/L were tried to determine the dose intolerable to tobacco. Callus could sustain in 10 mg/L kanamycin dose, but as the dosage was increased, calli turned white and beyond 40mg/L callus could not survive, indicating the limit of tobacco plants’ in built resistance against kanamycin. Explants, infected by pPMS clones (Agrobacterium), were directly selected in MS medium (1ppm NAA and 0.1ppm BAP along with kanamycin, 50μg/ml). Through a severe screening
**Plasmodium falciparum** (Genomic DNA)

5' CGC GGA TCC ATG TTA AAC ATT TCA CAA CAC CAA TGC 3'
5' CAA GAG CTC AGT TAG AGG AAC TGC AGA A 3'

Amplified fragment

BamHI/SacI

msp1<sub>19</sub>

nptII(Kan')

pBI121

BamHI

SaI

gus

Fig.9 Preparation of the Plant transformation vector pPM121
from 300 calli, around 190 (i.e. 65%) were selected, which could survive in the kanamycin medium. Calli were transferred to a new medium for shoot induction and around 100 shoots developed in new medium. Among the 100 shoots, few of them were abnormal, since they produced stunted plantlets, leafy structures. Rest of the healthy and normal plants were analyzed by PCR, which showed positive (60 plants) result and were transferred to liquid MS (containing 1 ppm IAA) for the root initiation. After 2 weeks when the roots were well developed, plants were transferred to Hoagland’s medium. Subsequently, full grown plants which had established rooting systems were transferred to a pots and then to field (Fig. 10).

Field transferred plants were further analyzed for the molecular presence of msp1, gene and its protein product. Throughout the regeneration process, ie. prior to field trial, media were always supplemented with kanamycin (50mg/L) and cefotaxime (250mg/L) for selection of transformants as well as to restrict Agrobacterium contamination.

**Table 5** Comparative study of callus as well as shoot formation in different combination of BAP and NAA (ppm). Values are given as the mean of 3 replicates.

<table>
<thead>
<tr>
<th>BAP/NAA (ppm)</th>
<th>0.5/0.1</th>
<th>0.5/0.5</th>
<th>1/0.5</th>
<th>1/0.1</th>
<th>2/0.2</th>
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<tbody>
<tr>
<td>Weeks</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>4.0 ± 0.2%</td>
<td>4.0 ± 0.6%</td>
</tr>
<tr>
<td>2</td>
<td>4.0 ± 1.2%</td>
<td>—</td>
<td>4.0 ± 0.6%</td>
<td>24.0 ±1.3%</td>
<td>20.0 ± 1.5%</td>
</tr>
<tr>
<td>3</td>
<td>20.0 ± 2.4%</td>
<td>10.0 ± 3.2%</td>
<td>24.0± 2.8%</td>
<td>80 ±4.2%</td>
<td>76.0 ±3.2%</td>
</tr>
<tr>
<td>4</td>
<td>64.0 ± 2.7%</td>
<td>50.0 ± 4.1%</td>
<td>68.0 ± 3.6%</td>
<td>88.0 ±2.9%</td>
<td>84.0 ± 4.8%</td>
</tr>
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</table>
Fig. 10 Different growth stages of pPM121 transformed plant.
A. Shoot emergence from callus; B. Plant regeneration in laboratory
C. Transgenic plant in field
Results

Molecular analysis of the transformed and regenerated plants

PCR analysis

Transformed plants were analyzed for msp1_{19} gene by PCR using above mentioned primers. Almost all plants showed positive fragment and ensured the presence of msp1_{19} gene. DNA of pPM121 was also taken along with the plant DNA as a positive control and bands from all of them appeared at the same position (Fig. 11), thus ensuring the presence of msp1_{19} fragment in transformed plants. As shown in Fig. 11, most of the plants showed clear bands at around 300 bp, which was nearly to the size of msp1_{19} gene (280bp). Although clear bands were found in transformed plants, untransformed plants did not show any trace of amplification (Fig. 11). Thus PCR analysis proved that msp1_{19} gene has integrated into the genome of transformed tobacco plants.

Southern analysis

Total DNA was isolated from transformed and untransformed plants. Dot blots as well as southern blots (hybridization) were performed to check the integration of msp1_{19} gene and its pattern in plant genome. Plant genomic DNA in a limited amount (1 \mu g) were blotted on nitrocellulose membrane and were hybridized with msp1_{19} fragment as probe. Many of the plants showed positive signals in varied intensities along with the pPM121 DNA (5ng), whereas nothing was found in case of untransformed plant DNA (Fig. 12A).

DNA from the experimental plants were also digested with restriction enzyme and southern hybridization was carried out. Undigested DNA while run on gel, blotted and hybridized with radiolabelled msp1_{19} gene, signals in the shape of blob were found in the radiogram at the same position (around 25kb) (Fig not shown). On the other hand lane loaded with untransformed plant DNA was found to be blank (no signal was found).
**Fig. 11** PCR analysis of T0 transformed plants to detect the presence of msp119 gene. Lane 1 and 13: 1 kb ladder and λ/HindIII markers, respectively. Lane 2: Untransformed plant. Lane 3-11: Transformed plants. Lane 12: DNA from pPM121 clone.
Fig. 12 A. DNA dot blot analysis of 6 transformed plants. DNA of pPM121 was taken as positive control. Untransformed plant has taken as negative control.

B. Southern blot analysis of NcoI/SacI digested 8 transformed plants. msp119 fragment used as positive control. Control: Untransformed plant DNA.
Southern hybridization was also performed with plant DNA from transformed and untransformed plants digested with BamHI/Sacl enzyme combination. As expected, the 280bp fragment was found when they were hybridized with msp119 fragment as probe (Fig. 12B). Known amount of msp119 fragment was loaded in the gel as control and to estimate the copy no. Result shows that msp119 gene is integrated in the genome of the transformed plants in different copy number. While most of the transformed plants showed the presence of msp119 gene, untransformed plants and even a few kanamycin resistant plants did not give any positive result.

Northern analysis

After confirming the presence of msp119 gene in transformed plants, northern analysis was performed to detect its transcription level in different plants. For this, RNA was extracted, blotted and then hybridization was performed using msp119 fragment as probe. Variable signals were observed among different plants, which might be due to the different level of transcription. Fig. 13 shows northern analysis with five different plants and also shows varied amount of RNA production, although each lane was loaded with almost equal amount of total RNA. The figure shows that plant no. 6 delivered considerably higher expression than plants no. 2, 3, 4 and 5. Few plants like no. 7, 8, 15, 18 which showed positive signals in PCR and Southern, did not however, produce any msp119 transcript. Untransformed plants showed no signal for msp119 gene.

Protein profile analysis

Based on northern analysis, selected transformed plants were further screened for the production of MSP119 protein. Plant derived MSP119 protein was tried to extract in different buffers and forms (as described in Protein extraction section of material and methods). Only the crude extract had been able to show clear bands with polyclonal antibody in western analysis (Fig. 14A). In another study, plant no 2 showed very low intensity signal. However,
Fig. 13  Northern blot analysis of 5 transformed plants. Total RNA were extracted from transformed and untransformed plants, fractionated in denatured formaldehyde gel and were hybridized to radiolabelled msp119. 
Lane 1: Untransformed plant. Lane 2: Plant no 2. Lane 3: Plant no. 3. Lane 4: Plant no. 4. Lane 5: plant no. 5. Lane 6: plant no. 6.
Lower panel signifies equal amount of RNA was loaded in each lane (10μg).

Fig. 14  Immunoblot detection of MSP119 protein in transgenic tobacco plants.
A. Cross-reactivity of plant derived MSP119 protein with polyclonal antibody raised in rabbit against bacterial (E. coli) MSP119 protein. Lane 1 and 3: Extracts from plant no. 4 and 6 respectively. Lane no. 2 and 4: Extract from untransformed plants. Lane 5: Bacterial protein (E. coli).
B. Cross-reactivity of transgenic plant derived MSP119 with monoclonal antibody G17.12. Lane 1: Bacterial protein (E. coli). Lane no. 2: Extract from plant no. 6. Lane no. 3: Untransformed plant extract.
few plants (3,5,11), that showed signals in both Southern and northern analyses, could not produce any detectable level of signal in western blot. After finding cross-reactivity of polyclonal antibody with some of the plant extracts, positive plants were further characterized by monoclonal antibody, G.17.12. Monoclonal antibody showed a very good cross-reactivity with the extract from plant no. 6 (Fig. 14B). Other plants like no. 2, 4, 10 have shown faint bands with G.17.12 monoclonal antibody.

Expression and estimation of MSP1_{19} was also confirmed by sandwich ELISA assays. Two different monoclonal antibodies (G.17.12 and 2F.10) were used in these assays. Expression level of recombinant MSP1_{19} varied considerably among all the five transgenic plants with both the monoclonal antibodies (Fig 15A and B). When the OD values were calculated in terms of the total soluble protein percentage, the MSP1_{19} protein produced by the transformed plants were fairly low, approximately 9-35 ng/mg of total soluble protein (Fig. 16). Plant no. 6 showed the best level of expression with both the monoclonal antibodies, which represented 0.0035% of the total soluble leaf protein. Plant no. 4 also showed a very good response with 2F.10 (15B), but with G.17.12 the cross-reaction was found to be less (15A).

**PCR analysis of T1 generation**

Although all the transformed plants flowered, not all of them produced viable seeds. Even germination percentage of the seeds was found to be very low. Seeds from the transgenic plants were germinated in the MS medium along with kanamycin (100mg/L). Plants, which grew well in the kanamycin media, were selected and eventually DNA was isolated from them. PCR analysis was performed to detect the presence of msp1_{19} gene in T1 progeny. Not all, but few of them from line 4,6 and 10 showed the presence of the transgene in the T1 plants (Fig. 17).
**Fig. 15** Detection of MSP$_{19}$ production in transformed plants by sandwich ELISA. C: Untransformed plant extract. No2, No4, No6, No10, No12: 5 transformed plants A. Detected by monoclonal antibody G.17.12. B. Detected by monoclonal antibody 2F.10

**Fig. 16** Production of MSP$_{19}$ protein in transgenic plants as measured by Sandwich ELISA with the help of two monoclonal antibodies, G.17.12 and 2F.10. C: Untransformed plant. 2, 4, 6, 10 and 12 are 5 transgenic plants.
Fig. 17 Confirmation of genetic inheritance of msp19 gene in T1 plants. PCR was performed with the DNA from T1 plants germinated from the seeds of pPM121 transformed T0 plant 4 and 6. 4.1 is the T1 generation of plant no 4; 6.1 and 6.2 are two T1 plants from plant no 6. P: pPM121 DNA was used as positive control.
Results

B. VECTOR SYSTEM II

Higher expression of MSP1\textsubscript{19} protein in tobacco plant with the help of Endoplasmic Reticulum signal

Since expression level in the earlier study was found to be low, for further enhancement of the plant produced protein a new vector system was tried based on the observation made by Haq et al (1995). Haq et al. found that the pIBT.201 vector facilitated enhanced amount of enterotoxin protein (LT-B) production in tobacco as well as in potato plants.

Construction of an improved vector for enhanced expression of MSP1\textsubscript{19} protein

New set of primers were designed (Fig.18) with an aim to clone the gene in a new vector pIBT.201 (kindly supplied by Dr. Mason, Boyce Thompson Institute, New York, USA) and also to tag an endoplasmic reticulum retention signal at the C-terminal end of the protein.

| 5’CAT GCC ATG GTA AAC ATT TCA CAA CAC CAA TGC 3’ | Forward primer |
| 5’CAA GAG CTC ATA GCT CAT CTT TCT CAG AGG AAC TGC AGA AAA TAC C 3’ | Reverse primer |

Fig. 18. Newly constructed primers designed for plant transformation.

In that direction, forward primer was designed with a Ncol restriction site at the 5’ end, where as reverse primer was designed with 18 base pair endoplasmic reticulum signal sequence, a stop sequence and a SacI site. The new set of primers was used to amplify and clone the msp1\textsubscript{19} gene from the parasitic
genome into pGEM-T vector. The clones were screened by blue-white selection (X-gal/IPTG), DNA was isolated and digested with a combination of Ncol/Sacl. Next, the sequence (~290bp) was cloned in multiple cloning site of a new vector called pIBT.201. Newly constructed clones were termed as pLS clones, which were 4.3 kb in size. pLS clones when digested with EcoRI/HindIII combination, they gave rise to two bands of 2.6 kb and 1.7 kb size, instead of 2.6 kb and 1.4 kb originated from actual pIBT201 (due to double digestion by EcoRI/HindIII combination) (Fig. 19A). Whereas, when it was digested with Ncol/Sacl enzyme combination, products gave rise to original pIBT201 vector (4.03kb) along with a 0.3 kb fragment of msp119 (Fig. 19B). The newly constructed pLS clones have a complete sequence of 35S CaMV promoter and TEV enhancer element adjacent to the kozak sequence (CCATGG) and msp119 gene along with SEKDEL sequence at the C-terminal end (ER retention sequence) and a stop codon (altogether 1.7 kb). Further, the complete cassette from the 35S CaMV promoter to the stop codon, was cloned in a promoterless plant transformation vector pBI101. For that, the 1.4 kb EcoRI/HindIII fragment from the pBI101 vector was cut out and complete cassette of 1.7 kb from pLS clone was inserted in the vector giving rise to 11.8 kb newly constructed pBILS clones. pBILS clones consist of kanamycin gene under the nos promoter along with nos-terminator as a selection gene and msp119 gene under a strong CaMV promoter-TEV enhancer complex along with ER retention signal. Further, the pBILS clones were transformed in the disarmed Agrobacterium strain, LBA4404 and the positive clones, selected in kanamycin as well as in spectinomycin, were termed as pPRL clones. Eventually the pPRL clones were used for tobacco plant transformation (Fig. 20).

Fig 21 compares two plant transformation vectors pPM121 and pPRL used for two sets of tobacco transformations. pPRL has an additional promoter enhancer sequence from tobacco Etch virus, situated just after the 35S CaMV
Fig 19. A. Lane 1, 8: XVII and λ/BstEII markers. Lanes 2-5: EcoRI/ HindIII digested pLS clones. Insert: EcoRI/HindIII digested fragment of pLS clone. Lane 7: pIBT.201 (EcoRI/HindIII digested). B. Lane 1-2: pLS1 and 2. Lane 3-4: pLS1 and 2 (NcoI/SacI digested). Lane 5: XVII marker. 0.3kb msp1.19 fragments are marked by arrows.
**P.falciparum G-DNA**

PCR amplification AND Cloning

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5'CAT GCC ATG GTA AAG ATT TCA CAA CAC TGC 3'
5'CAA GAG CTC ATA GCT CAT CTT TCT CAG AGG AAC TGC AGA AAA TAC G 3'
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<table>
<thead>
<tr>
<th>5' CAAGAG CTCATA GCT CAT CTTTCTCAG AGGAAC TGC AGAAAA TAC C 3'</th>
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Both digested with NcoI/Sacl and ligated

<table>
<thead>
<tr>
<th>plBT201(4.05kb)</th>
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<tr>
<td>pLS(4.35kb)</td>
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<tr>
<td>pBILS (11.8 kb)</td>
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E- EcoRI
H- HindIII
S- Sacl
N- NcoI

**Fig. 20** Schematic representation of the cloning and preparation of pPRL clones containing msp119
Fig. 21 Comparison of two constructs used for the plant transformation towards expression of msp1_{19} gene in tobacco plants. pPM121 was used earlier and found out to be less effective. pPRL construct is a newly developed construct featuring enhancer sequence. H- HindIII; E- EcoRI; X- Xhol; N- Ncol; S- SacI
Results

promoter. Another additional feature in pPRL vector is the endoplasmic reticulum signal (SEKDEL) at the C-terminal end of the msp1<sub>19</sub> fragment.

Confirmation of the msp1<sub>19</sub> in plant transformation vector (pPRL clones)

To ensure the presence of parasitic gene (msp1<sub>19</sub>) in the pPRL plant transformation vector several southern hybridizations were performed using msp1<sub>19</sub> fragment as probe. Transformed positive clones were run in gel in undigested as well as digested conditions. All undigested clones showed band at a quite high position (~23kb) (Fig. 22 A and B). pPRL3 and pPRL6 clones were developed by the transformation of pLS3 and pLS6 clones in LBA4404. HindIII digested pPRL clones showed band at around 12 kb while EcoRI/HindIII digested DNA showed a single band at around 1.7 kb, which is the original size of the complete cassette containing msp1<sub>19</sub> gene (Fig. 23A). While the pPRL clones were double digested with Ncol/Sacl restriction enzyme and were hybridized with msp1<sub>19</sub> gene band was found at around 0.3 kb position, which was exactly the sizes of msp1<sub>19</sub> gene used as positive control (Fig 23B). The result assured the presence of msp1<sub>19</sub> gene in the pPRL Agrobacterium clones. Further step was to clone the gene in tobacco plant.

Tobacco plant transformation with msp1<sub>19</sub> gene and regeneration process

Following co-cultivation, explants were directly transferred to kanamycin (50 mg/L) selection plates along with cefotaxime (250mg/L) and the selection was started at the primary stage. In earlier selection media, which was used for the previous transformation (with pPM121 clone) i.e., the combination of 1ppm BAP and 0.1 ppm NAA was found to be very effective for the callus induction as well as for early shoot initiation. Out of 400 explants infected, 250 calli with small protrusion of shoots were observed within 4 weeks. Around 62.5% explants were healthy i.e., green in color. Repeated transfer of the calli material in regular interval (15 days) was found to be effective as shoots started growing fast. Shoots started sprouting from the calli and were
Fig. 22 Southern analysis of undigested Agrobacterium clones.
A. pPRL3 clones. B. pPRL6 clones. LBA4404: Untransformed Agrobacterium as negative control.
Fig. 23 A. Southern analysis of digested pPRL.6.8. Lane no 1: λ/HindIII used as marker. Lane no 2: purified msp19 fragment. Lane no 3: LBA4404 as negative control. Lane 4: pPRL.6.8 DNA cut by HindIII. Lane no 5: pPRL.6.8 is double digested with EcoRI/HindIII. B. Southern hybridization of NcoI/SalI digested pPRL clones. Control: Untransformed LBA4404. Lane no 3 and 4: pPRL.6.8 and 6.9 DNA digested with NcoI/SalI combination
transferred to a new MS media containing 1 ppm IAA and 0.6% agar. The plantlets sufficiently grown (8-10cm) along with developed roots were first transferred to Hoagland’s media for 7 days and then to ½ MS (without sucrose) media for getting well regenerated plants. After one week, plants were first transferred to pots and subsequently to the field (Fig. 24). During the whole process, plants were always challenged against kanamycin (50mg/L) and cefotaxime (250 mg/L) to restrict the growth of Agrobacterium.

**Molecular analysis of the pPRL transformed plants**

**PCR analysis of the transformed plants**

Leaves were taken from the small plantlets and genomic DNA was isolated from the plants. First step of confirmation for the presence of msp119 gene in transformed and regenerated plants was performed by PCR analysis with the help of newly constructed primers (Fig. 25). DNA was isolated from plantlets as well as from well-grown plants. PCR was performed with those DNA and was found that maximum plants have shown bands in agreement to the size of msp119. PCR was done at the different stages of plant growth stages to confirm the absence of Agrobacterium contamination. PCR positive plants were further analyzed for the integration pattern, transcript formation and protein expression.

**Southern hybridization analysis**

Southern hybridization was performed to confirm the presence of msp119 gene in the transformed plants. Genomic DNA digested with different restriction enzymes like EcoRI, HindIII (Fig. 26A) and BamHI were run in 0.8% agarose gel, blotted and then hybridized with msp119 radiolabelled probe. Transformed plants showed different pattern of bands in the southern blots. Presence of more than one band in the same lane showed different copy no. as well as the variation in transgene integration in the plant genome (Fig. 26B). Plant no. 2, 18 and 23 showed single gene integration. While plant no. 2 showed the band at 4.0 kb size, plant no 18 and 23 showed band at a higher position (5 kb).
Fig. 24 Different growth stages of pPRL transformed plants. A. Growing callus in selection medium. B. Sprouting shoots. C. Transgenic plant in laboratory. D. Transgenic plant in field.
Fig. 25 PCR analysis of T0 transformed plants to detect the presence of msp19 gene. Lane 1: Untransformed plant. Lane 2-8: Transformed plants. Lane 9: λ/BstEII marker
Fig. 26  Southern blot analysis of the transformed plants for the presence of msp1_{19} gene. A. Total G-DNA fully digested with HindIII. Control: Untransformed plant. Lane 2-8: G-DNA from seven PCR positive plants were run in 1% agarose gel. In each lane 20 μg DNA was loaded.
A. Southern hybridization of the HindIII digested plant DNA with msp1_{19} fragment as probe. Control: Untransformed plant. Lane 2-8: 7 transformed plants
Results

Plant no. 5, plant no. 13 and plant no. 17 showed double integration, whereas plant no. 2 showed integration at position 3.1 kb and 9.4 kb, plant no. 13 showed 5.2 kb and 9.4 kb, and plant no. 17 showed gene integration at 2.2 kb and 3.2 kb. Plant no. 21 rather showed that msp119 gene has been incorporated at 4 different places in the plant genome, which are 9.5 kb, 5.5 kb, 3.6 kb and 2.3 kb.

Northern hybridization analysis

Detecting the presence of msp119 gene in the transformants, next step was to find whether proper transcripts could be achieved. Therefore, 15μg total RNA was isolated and loaded in the formaldehyde gels in equal amount, gels were blotted and blots were hybridized with radiolabelled msp119 fragment. Many of the PCR and Southern positive plants showed clear, definite and positive signals in the blots, but in no case untransformed plants showed any trace of signal (Fig. 27). Signal varied with transformed lines, although same amount of RNA was loaded to each and every lane (Fig. 27 A and B). Fig 27 shows that plant no 2, 13, 18 and 23 produced relatively higher level of transcript than the others. Plants, which showed clear signals, were screened and in the next step protein analysis was performed.

Protein analysis

Several buffers were tried for the extraction of plant expressed parasitic protein (MSP119). Earlier, heterologous protein was tried to extract in buffer containing 0.1M sodium phosphate (pH 7.4), 0.1M sodium ascorbate, 0.1% Triton X-100 and 2mM phenylmethylsulphonyl fluoride, but it was not found to be efficient. New buffer used for the extraction (Protein extraction section in Materials and Methods) was found more effective. Concentrated plant extracts were used to perform western analysis to detect MSP119 protein. Since we have loaded crude plant extracts, primarily ECL kit was used to detect the bands. Clear bands were found when cross-reacted with polyclonal antibody (Fig. 28). Plants no 13, 18 and 23 have shown relatively more amount of
Fig.27 Northern blot analysis of pPRL6.8 transformed plants. 10µg RNA was loaded in each well, blotted and then hybridized with msp119 fragment as probe. A. Control: Untransformed plant. 6, 14, 17, 23 are different transformed plants. B. Control: Untransformed plant; 13, 15, 18, 21 are different transformed plants; Lower block signifies equal amount of RNA was loaded in each lane.
Fig. 28 Western analysis of transformed plants (by ECL kit). Transformed plant extracts were run in 15% SDS-PAGE and blotted. M: Low mol. wt. marker (Gibco BRL). BP: Purified protein from *E. Coli*. Plant proteins were cross-reacted with polyclonal antibodies raised in rabbit against MSP1$_{19}$.

Fig. 29 Western blot analysis of transformed plants cross-reacted with monoclonal antibody G17.12. BP: Purified bacterial MSP1$_{19}$. Lane2-6: 5 pPRL transformed plants. C: Extract from untransformed plant.
MSP119 protein expression. Proteins extracted from all the northern positive plants were unable to cross-react with polyclonal antibodies raised against MSP119. Whereas many of them have shown clear bands, indicating the presence of sufficiently good amount of MSP119 protein. Concentrated extracts from untransformed plants did not show any evidence of presence of MSP119. After the cross-reactivity was achieved with polyclonal antibody, monoclonal antibodies were also used to check the conformational aspect of the plant-derived protein. G17.12 (kindly supplied by S. Longcare, Switzerland), a monoclonal antibody was used at the first stage and it could react with the heterologous protein (fig 29). ECL kit was used to develop the western blot to achieve a clear and prominent band.

To quantify the protein, sandwich ELISA was performed with polyclonal as well as monoclonal antibodies. Two monoclonal antibodies, G17.12 and 2F.10 were used to confirm the heterologous MSP119 expression in plant. Their protein contents were measured by Bradford method. Estimation of plant produced MSP119 were based on ELISA reading of transgenic plant extracts to known amount of bacteria produced protein used as positive control. In both the cases it was found that pPRL transformed plants have increased the MSP119 expression level (Fig. 30 and Fig. 31). Maximum expression was found in plant no. 13, which showed 0.01% and 0.012% of total soluble protein by G17.12 and 2F.10 respectively. Where as old plant (plant no 6 of the pPM121 transformed plants) showed 0.0035% in both the cases.

To observe and evaluate whether plant derived MSP119 adopt a conformation which resembles that of the native antigens, ELISA was performed where sera obtained from different P. falciparum infected patients. Primarily MSP119 from transformed plant no 13 extract was cross-reacted with sera from different P. falciparum infected patients collected from All India Institute of Medical Sciences (Delhi). Cross-reactivity with the patient sera was found to be less as compared to that of the monoclonal antibodies. Sera from patients 3738, 3792 and 3879 could recognize the recombinant plant proteins well (Fig. 32A),
**Fig. 30** Sandwich ELISA was performed to detect the amount of MSP1_{19} produced in pPRL transformed plants. Plant extracts were crossreacted with monoclonal antibody G17.12. C: Untransformed plant extract. 2,5,6,7,13,17,18,23 are numbers of different transformed plants. Old plant: Plant extract from pPM121 transformed plant no 6

**Fig. 31** Sandwich ELISA was performed to detect the amount of MSP1_{19} produced in pPRL transformed plants. Plant extracts were crossreacted with monoclonal antibody 2F.10. C: Untransformed plant extract. 2,5,6,7,13,17,18,23 are numbers of different transformed plants. Old plant: Plant extract from pPM121 transformed plant no 6
**Fig. 32** Cross-reactivity of plant expressed MSP1<sub>19</sub> with *P. falciparum* infected patient sera with the help of sandwich ELISA. C: Untransformed plant extract. BP: Purified bacterial MSP1<sub>19</sub>. T: Transgenic plant extract.

A. Sera from AIIMS, Delhi. Csera1 and Csera2: Sera from healthy human being. 3738, 3746, 3792, 3779, 3879 are different patient sera

B. Sera from Rourkela, Orissa. Csera1 and 2: Sera from healthy human. Sera1-8: 8 different human sera

All data are mean of 2 experiments
whereas the other patient sera could hardly differ from the untransformed control plant extract. In another set of experiment, where 8 different sera from Rourkela (Orissa) were taken, recombinant plant protein performed differently with different sera. Sera from patient 2, 5, 6, 7 and 8 showed reasonably well cross-reaction, whereas other sera did not show much different cross-reactivity than the control (Fig 32B).

**Purification of plant produced MSP1\textsubscript{19} protein**

After confirming the presence of recombinant MSP1\textsubscript{19} protein in tobacco plants through western, next step was the purification of heterologous protein form plants. Crude plant extracts in extraction buffer were concentrated with the help of sucrose and concentrated extracts were used for purification. Dynal magnetic beads were used for the purification of plant expressed MSP1\textsubscript{19} (as described in Protein purification section of material and methods). During the process, affinity column purification principle (Dynabead kit) was used where mice antibody (1:1000 dilution) raised against MSP1\textsubscript{19} was primarily coated over magnetic beads and then used as a column to purify plant expressed MSP1\textsubscript{19} from the extract. Purified MSP1\textsubscript{19} was loaded in 15% SDS gel and was stained by comassie blue. Comassie blue staining could detect the presence of very light band of MSP1\textsubscript{19} probably due to low protein content. To get a stronger intensity of the band, a more powerful staining technique i.e. silver staining was performed and clear band was found visible in the stained gel (Fig 33). There was an additional light band found at the higher molecular weight level of plant no. 13, which could be due to the glycosylated product formed during the posttraslational period.

**Immunogenicity of orally fed transformed plants**

Five mice were fed with concentrated transformed plant extract. Three mice were also fed with untransformed tobacco plant. All the mice were given 3 booster doses of plant extracts each in 21 days interval. Each mouse was fed with 2.5 μg of MSP1\textsubscript{19} protein in each booster. After 21 days of the final
Fig. 33 Purified plant MSP1<sub>19</sub> protein was run in 15% native gel and was stained with silver nitrate. Silver nitrate stained gel shows presence of MSP1<sub>19</sub> protein in transgenic plants. Marker: Protein marker. No 13 and 17 are two transgenic plants; BProtein: Bacterial MSP1<sub>19</sub>. 
booster, blood was taken from the mice and ELISA as well as western blot analyses were performed to detect oral immunogenicity. Monoclonal antibody, G17.12 was used to detect the antibody raised against plant derived MSP1_{19} in mice. Transgenic plant fed mice showed elicited immunogenicity, though untransformed plant fed mouse has showed some immunogenicity (Fig. 34) in ELISA. Different dilution of sera (1:10, 1:20, 1:40, 1:80, 1:160) were used in ELISA and it was found that after 1:40 dilution the reactivity got reduced with the increasing dilution.

In the next step all the sera were used to perform western blot analysis against purified recombinant bacterial MSP1_{19} protein. Sera from four mice, which were fed with extract from plant no 13 were cross-reacted with 5μg purified bacterial MSP1_{19} protein at 1:50 dilution. While as positive control, bacterial recombinant protein was cross-reacted with 1:1000 dilution of mouse anti-MSP1_{19} (Fig. 35).

**PCR analysis of T1 generation**

Transgenic plants, which were grown in the field, produced flowers. Seed viability was checked and found to be very low. Seeds were collected in large amount and tried to germinate in MS medium along with kanamycin (100μg/ml). Maximum seeds were found to be nonviable. Germination percentage was also found to be very low. Seedlings, which have grown in the selection medium were selected and DNA was isolated from the plants. PCR was performed to detect the presence of msp1_{19} gene in T1 progeny (Fig. 36).
Fig. 34  Orally fed mice sera were cross-reacted with bacterial MSP1₁₉ (purified) to detect the immunogenicity of the plant expressed protein through oral feeding. A. Sera from four mice fed with transgenic plant extract. Control: Sera from mouse fed with untransformed plant. No1-4: Sera from 4 mice. B. Sera from purified MSP₁₁₉ injected mice which was used as positive control.
**Fig. 35** Western analysis of mice sera, which were orally fed with plant no 13 extract. 5μg *E. coli* purified MSP1<sub>19</sub> protein were loaded in each lane. M: Protein marker. BP: Cross-reacted with mouse anti-MSP1<sub>19</sub> sera. No.1-4: Cross-reacted with sera from 4 different mice fed with transgenic plant extract. C: Cross-reacted with sera from mouse fed with untransformed plant extract.

**Fig. 36** PCR analysis of few T1 plants from plant no 13. C: Untransformed plant. 1-7: 7 T1 plants which confirms inheritance of msp1<sub>19</sub> gene in the next generation.