RESEARCH ARTICLE

Development of transgenics in Indian oilseed mustard (*Brassica juncea*) resistant to herbicide phosphinothricin

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Transgenic lines resistant to herbicide phosphinothricin (PPT) were developed in mustard (*Brassica juncea*), a major oilseed crop grown in more than 6 million hectares of land in North India. Seedling-derived hypocotyl explants were transformed with a disarmed *Agrobacterium tumefaciens* strain GV3101. The developed constructs contained the *bar* gene encoding the enzyme phosphinothricin-acetyl-transferase (PAT) which inactivates phosphinothricin (PPT) by acetylating it. The expression of the *bar* gene was controlled either by the double enhancer version of CaMV35S promoter (35Sde*bar*) or a CaMV35S promoter with a leader sequence from RNA4 of alfalfa mosaic virus introduced at the 5′ end of the *bar* gene (35SAMVL*bar)* or without (35S*bar*) it. Plant viral leader sequences have been shown to be translational enhancers. *In vitro* selections for transformed plants were carried out on a medium containing PPT. Transgenic shoots were recovered at a frequency of 23% with 35Sde*bar* gene construct and at a frequency of 16% with 35SAMVL*bar* containing construct. Transformation frequencies were low with 35S*bar* construct. Individual transgenics with 35Sde*bar* and 35SAMVL*bar* constructs were tested for copy number on both the right and left border flanks of T-DNA by Southern hybridization. Single copy transgenic lines were further analysed for transcript levels of the *bar* gene by Northern blotting and for protein levels by PAT assays. Wide variation in expression levels were observed, particularly amongst the transgenics containing the 35Sde*bar* construct. Single copy transgenics were selfed to develop homozygous lines which could be used for the study of resistance to herbicide PPT at the field level and to correlate this protection with expression levels observed through molecular analysis. Herbicide-tolerant lines could be used for testing the possibility of low-till or no-till cultivation of mustard in the rain-fed areas where it is extensively grown.

WE report in this study development of transgenic mustard (*Brassica juncea*) plants that are resistant to herbicide phosphinothricin (PPT). The use of herbicides is an integral part of the mechanized agriculture being practised in Western countries. In India herbicides are currently being used extensively for weed control in wheat and rice crops. In view of the increasing concern about pollution of the environment, toxicity to animals and persistence of the herbicides or their residues in the soil and water, herbicides like glyphosate and PPT which are highly effective at low dosage, safe for animals and rapidly degraded in soil could be more useful for weed control[1]. However, these herbicides are non-selective and therefore have to be used in conjunction with transgenic crops that are resistant to these herbicides. Globally, crops with resistance to non-selective herbicides occupy about 19.8 million hectares out of the total 27.8 million hectares under transgenic crops[2]. In India, a major benefit of herbicides could be in no-till or low-till agriculture for moisture conservation in the rain-fed areas and for multiple cropping in the irrigated intensively cultivated areas.

DL-phosphinothricin also known as glufosinate is a broad spectrum, post-emergent, contact herbicide (with some systemic activity), which is marketed as Basta (Agrevo). Glufosinate is a truncated version of bialaphos which is a naturally occurring herbicidal tripeptide antibiotic 'L-phosphino thricyl-L-alanyl-L-alanine' produced by *Streptomyces hygroscopicus*[3]. Glufosinate lacks the terminal alanine and contains L-phosphinothricin (PPT) which is the active herbicidal ingredient.

PPT, an analogue of glutamate is a potent inhibitor of glutamine synthetase (GS)[4] – an essential enzyme in the assimilation of ammonia and in the regulation of nitrogen metabolism in plants. The inhibition of GS by PPT leads to rapid accumulation of intracellular ammonia, cessation of photorespiration and photosynthesis leading to cell death[5]. Though there are several isoenzyme forms of GS[6], PPT is known to inhibit all identified forms. PPT-based herbicide 'Basta' is an ideal herbicide, as it has high unit activity, and is effective against a wide variety of plant

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species. The herbicide is environmentally safe as it is non-toxic and rapidly degraded, resulting in minimal accumulation in the soil[7].

Genetically-engineered resistance to PPT has been developed internationally in a wide range of dicotyledenous and monocotyledenous crops, either by overproduction of the target enzyme, GS[8] or by detoxifying PPT[9–11]. The latter strategy has been highly successful and has been achieved by the expression of an enzyme phosphinothricin-*N*-acetyl transferase (PAT) which inactivates PPT by converting it to *N*-acetyl-L-phosphinothricin in the presence of acetyl CoA. PAT is encoded by the *pat* gene from *Streptomyces viridochromogens*[12] and the *bar* gene isolated and characterized from *S. hygroscopicus*[13]. The two genes share a homology of about 87% (ref. 12), but of the two, the *bar* gene has been used more extensively for the development of transgenic plants resistant to PPT.

In this study transgenics of *B. juncea* were developed using the *bar* gene with different versions of cauliflower mosaic virus (CaMV35S) promoter. Transgenic plants have been characterized for their expression levels both at the transcriptional and translational levels. T_0 plants were selfed to develop homozygous lines which could now be used for field level analysis to correlate expression with extent of resistance to PPT at field level. *B. juncea* (Indian mustard) is a major oilseed crop of the Indian subcontinent being cultivated as a winter crop mainly in the northern parts of the country in around six million hectares of land[14]. Transgenic lines developed in this study would allow testing the possibility of low-till cultivation of mustard both for moisture conservation and for the development of two-crop systems in the rain-fed and low-irrigation potential areas of North India.

Materials and methods

Vector constructions

Three different constructs containing the *bar* gene were developed in the binary vector pGSFR780A (ref. 11). In construct 1, the *bar* gene is under the transcriptional control of a CaMV35S double enhancer promoter (35Sde)[15]. In the 35Sde promoter the – 90 to – 340 region of the CaMV35S promoter has been duplicated at the – 340 region of the same. The 35Sde promoter was removed as a XbaI fragment from plasmid pRDF and cloned in pMCS5 (ref. 16). The *bar* gene was isolated as a SmaI fragment from the plasmid pDM302 (ref. 17) and cloned at the SmaI site in pBluescript SK^+ (Stratagene) upstream to a polyA signal of octopine synthase gene (ocspA) which was earlier cloned in as a EcoRI–HindIII fragment in pBluescriptSK^+. The *bar*–ocspA cassette was then cloned downstream to the 35Sde promoter as a BamHI–XhoI fragment. The 35Sde–*bar*–ocspA was then removed as a HindIII fragment, Klenow filled and cloned at the SnaBI–NruI site of the binary vector pGSFR780A to make the vector 35Sde*bar* (Figure 1 *a*).

For construct 2, the CaMV35S promoter (35S)[15] was removed as a HindIII–XbaI fragment from pRT105 (ref. 18) and cloned in pMCS5. The *bar*–ocspA cassette as described above was cloned downstream to the 35S promoter as a BamHI–XhoI fragment. The whole expression cassette flanked by HindIII sites was removed, blunt-ended by Klenow filling and cloned at the SnaBI–NruI site of the binary vector pGSFR780A creating the vector 35S*bar*.

A 42-bp long AMV leader[19] was synthesized by using two complementary oligonucleotides which on annealing generated a 42-bp long XbaI–BamHI DNA fragment. This was cloned in between the 35S promoter and the *bar* coding region in 35S*bar* creating a *bar* gene cassette transcriptionally fused to the AMV leader to develop the vector 35SAMVL*bar* (Figure 1 *b*). The binary vectors 35Sde*bar*, 35S*bar* and 35SAMVL*bar* thus developed were electroporated into *Agrobacterium tumefaciens* strain GV3101 (ref. 20), a disarmed nopaline strain.

Transformation of B. juncea

A. tumefaciens strain GV3101 containing a binary plasmid was grown overnight in 5 ml of liquid YEB supplemented with appropriate antibiotics. Around 1 ml of the overnight culture (OD_{600} 1.2) was inoculated in 30 ml of YEB medium without antibiotics and the culture was grown to reach an OD_{600} of 0.5–0.6. The cells were collected by centrifugation and suspended in N1B1 medium[21] (MS medium containing NAA 1 mg/ml and BAP 1 mg/ml) to obtain an OD_{600} of 0.3. These cells were then used for infection of hypocotyl explants following a modified protocol of Bade and Damm[22] which is described below.

B. juncea var RLM198 previously reported to be a highly regenerating genotype[21] was used for transformations. Seeds of the variety were maintained by selfing. Seeds were

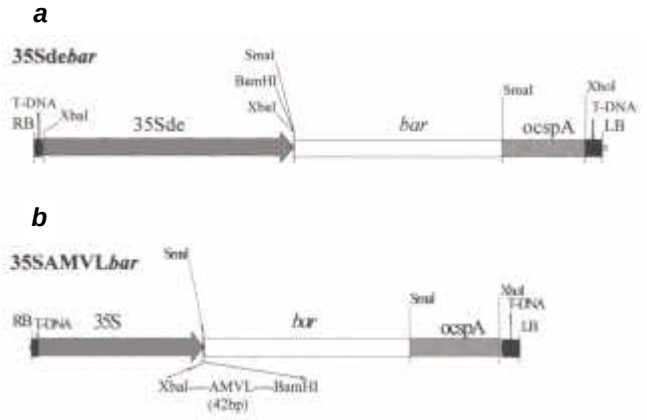

Figure 1. Constructs 35Sde*bar* (***a***) and 35SSAMVL*bar* (***b***) within the T-DNA borders used for developing phosphinothricin-resistant *B. juncea*. These cassettes were cloned in binary vector pGSFR780A for *Agrobacterium*-mediated plant transformation. The construct 35S*bar* is the same as 35SAMVL*bar* except that it lacked the AMVL sequence between the XbaI and BamHI sites.

germinated aseptically in glass tubes (4–5 seeds per tube) on MS[23] medium in dark for 1 day, then transferred to light (200 lux, 16 h light, 8 h dark) and maintained at 23 ± 1°C for 5 days. Approximately 300 hypocotyl explants (0.5–1.0 cm) from the 6-day-old seedlings were cultured in a 500 ml Erlenmeyer flask containing ~ 80 ml liquid N1B1 medium on a shaker (100 rpm) in diffused light for ~ 24 h. The liquid medium was removed from the pre-cultured explants and subsequently the explants were incubated in ~ 50 ml bacterial suspension for 30 min in stationary condition. After infection, the bacterial suspension was removed and fresh N1B1 medium (~ 80 ml) was added and co-cultivation was carried out on a shaker (100 rpm) till the medium became slightly turbid (around 18 h growth). Explants were rinsed twice for 1 min with 25 ml of the WM medium (N1B1 medium containing 200 mg/l augmentin, Time Cap Pharma Lab Pvt Ltd) and twice for 30 min with 80 to 100 ml of WM medium on a rotary shaker (at 100 rpm). The WM medium with all the explants was carefully poured in a petri dish and the medium was removed with a pipette. Excess bacterial suspension was removed by placing the explants on a filter paper and the explants were plated on SIM medium (N1B1 medium containing augmentin 200 mg/l, $AgNO_3$ 20 μM and PPT 10 mg/l, and solidified with 0.8% agar, HiMedia RM301). Green plantlets that differentiated on the SIM medium were transferred to the MS medium with IBA 2 mg/l, PPT 10 mg/l and augmentin 200 mg/l for rooting. Only one shoot from each explant was transferred in this manner. Following rooting, which took place within 15–20 days, the shoots were subcultured at monthly intervals on the same medium till they were transferred to the soil.

Plants were transferred in the month of November to the field in a net-house built according to the guidelines laid down by the Department of Biotechnology, Government of India. The plants were selfed as well as back-crossed to generate homozygous lines and T_1 heterozygous plants, respectively.

Molecular analysis of transgenic lines

For the analysis of copy number of insertions in transgenic plants by Southern analysis, total DNA was isolated from leaves of field grown plants by the CTAB method[24]. DNA (10 μg) was digested with either BamHI or XhoI and electrophoresed on a 0.8% agarose gel. DNA fragments were blotted onto nylon membrane (Hybond N^+, Amersham), followed by hybridization with probe DNA which had been labelled with [^{32}Pα] dCTP using the Megaprime DNA labelling system (Amersham Pharmacia Biotech). A SmaI fragment containing the *bar* gene (~ 0.55 kb) was used as the probe (Figure 1).

For Northern blot analysis total RNA (20 and 40 μg) isolated from leaves was electrophoresed on a formaldehyde agarose gel, blotted onto a nylon membrane (Hybond N^+, Amersham) and hybridized with ocspA fragment (Figure 1). The same blots were reprobed with a polyubiquitin gene probe taken from the plasmid pUBQ4 (ref. 25) to normalize differences in quantities of RNA loaded on different samples. The hybridization signals were quantitated on a phosphorimager (Fuji Photofilm Co Ltd, Japan).

PAT activity was determined after acetylation of PPT with ^{14}C-acetyl CoA by thin layer chromatography[10] (silica TLC plates from E. Merck, India). In the presence of PAT the radio-labelled acetyl group is transferred to PPT, giving a ^{14}C-labelled *N*-acetyl-PPT. The PAT activity was performed at different concentrations of protein extracts to enable calculation of the PAT activity in the linear range. The levels of unutilized ^{14}C-acetyl CoA and acetylated PPT product were measured following phosphorimaging of the chromatogram. PAT activity is represented as % conversion per μg of the protein (i.e. signal for ^{14}C-acetyl–PPT/signal of ^{14}C-acetyl-PPT + unutilized ^{14}C-acetyl CoA).

Results and discussion

Genetic transformation of var RLM198 with 35Sdebar, 35Sbar and 35SAMVLbar constructs

B. juncea var RLM198 hypocotyl explants were transformed with binary vectors containing 35Sde*bar*, 35S*bar* and 35SAMVL*bar* constructs. Out of the 1502 explants infected with 35Sde*bar*, 344 explants regenerated (overall frequency of around 23%). In infections with *Agrobacterium* containing 35SAMVL*bar* construct, out of 1988 explants infected, 391 produced shoots (15.8% regeneration frequency). However, in experiments with 35S*bar* construct we observed only 6% transformation frequency (Table 1). Callusing and regeneration time varied for the three different constructs; for 35Sde*bar* construct, regeneration started in 20 days while with the constructs containing 35S promoter, regeneration started in 30 days. Of the regenerating explants, 150 transgenics with 35Sde*bar* construct and 65 with 35SAMVL*bar* construct were transferred to the field. Individual transgenic plants that were transferred to a containment net-house, grew and flowered normally and set copious seeds like non-transformed plants. Only a few plants were obtained with the 35S*bar* construct. Transgenic plants with the 35Sde*bar* and 35SAMVL*bar* constructs were further characterized.

Table 1. Percentage of transformation observed with *B. juncea* cv. RML198 with 35SAMVL*bar* and 35Sde*bar* constructs

Construct	No. of experiments done	Total no.of explants used	Mean % transformation ± SE*
35Sde*bar*	4	1502	23.00 ± 4.96
35S*bar*	4	2602	6.04 ± 0.47
35SAMVL*bar*	6	1988	15.80 ± 1.68

*Percentage transformation was calculated as the number of explants giving rise to shoots over the total no. of explants used.

Identification of single copy 35Sdebar and 35SAMVLbar plants by Southern analysis

Transgenic plants transferred to soil were analysed by Southern hybridization for finding the copy number and pattern of transgene integration. Transgenes are known to integrate in the plant genome either as single copies or as multiple copies. During multicopy integrations, the copies may be linked (in the form of repeats) or may be unlinked. Therefore, it is imperative that both the genomic flanks of the transgene marked by the left border (LB) and the right border (RB) of the T-DNA be analysed. In the present study this was carried out by Southern hybridization of BamHI as well as XhoI digested genomic DNA of independent transgenic lines with the SmaI digested *bar* gene fragment as a probe (Figure 1). The analysed BamHI digestion indicated the number of copies toward the LB while RB flanks were analysed by XhoI digests. Figure 2 represents the hybridization patterns observed with some of the transgenics containing 35Sde*bar* construct. Of the 46 transgenics containing 35Sde*bar* construct and screened by Southern hybridization, 7 were found to be single copies. In case of 35SAMVL*bar* transgenics, 10 out of 50 T_0 plants had single copy insertion. The other lines had two or more copies (Table 2). Single copy integration events were thus observed at a frequency of around 15–20%.

Bar gene transcript levels in single copy 35Sdebar and 35SAMVLbar transgenic lines

Transgenics with single copy integrations were analysed for the expression of the *bar* gene. Levels of *bar* transcripts were quantitated by Northern hybridization of total RNA with ocspA as the probe. To normalize differences in quantities of RNA loaded between different samples, the blots were reprobed with a polyubiquitin gene probe. Further, to allow a comparison of transcripts levels in different blots, a common RNA sample (that of plant 1A, transformed with 35Sde*bar* construct, whose level was taken to be 100 arbitrary units) was loaded in all the gels. The signals obtained from the other samples were represented in comparison to that of 1A. The transcript levels observed in the different transgenic lines are represented in Figure 3 *a* and representative Northern blots are shown in Figure 3 *b*.

Table 2. Distribution of copy number of transgene integrated in transgenic plants with two different constructs

Construct	No. of plants analysed for copy numbers	No. of plants with one copy of transgene	two copies of transgene	more than two copies of transgene
35Sde*bar*	46	7	15	24
35SAMVL*bar*	50	10	7	33

The transcript levels observed in different lines with 35Sde*bar* varied over 19 folds, line IE having the lowest and ID the highest transcript levels. The variations observed in the 35SAMVL*bar* transformed lines were significantly less, the difference being only approximately 3.7-fold between the lowest and highest expresser. Variations in transgene expression in different lines is a well-recorded phenomenon, variations being up to 100-fold and more[26]. This variation could be either due to the copy number of

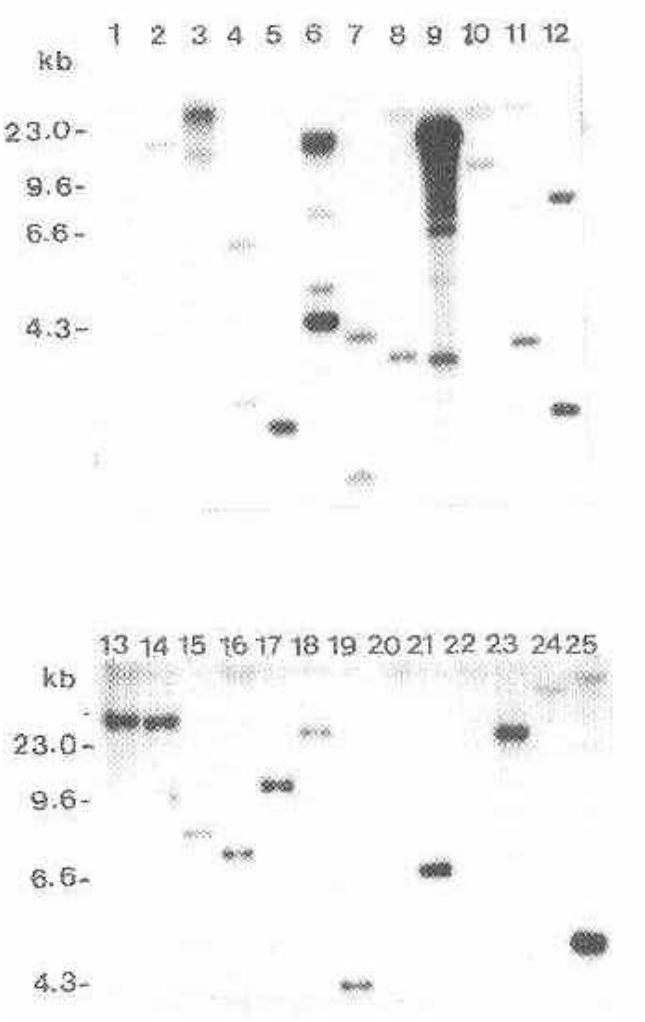

Figure 2. Southern analysis of transgenic lines generated with 35Sde*bar* construct. DNA from individual plants were digested with BamHI (lanes1 to 12) and hybridized to radiolabelled *bar* gene as the probe. DNA of putative single copy plants as identified from the BamHI blots was digested with XhoI (lanes 13 to 25) and probed for copy number towards the right border of T-DNA. For example, transgenic plant analysed in lanes 2 and 15 have single copy integration while in some cases though a single band is observed when probed at one of the borders (lane 23), two copies are observed on the other border (lane 12).

transgene or due to its site of integration[27]. As in this study only plants with single copy integration events have been analysed, the observed variation in expression can be attributed only to the influence of the flanks on the transgene, known as 'position effect'[28]. On *Agrobacterium* infection, the T-DNA integrates randomly in the plant genome and while it has been observed that most integrations are at transcriptionally active sites[29], the variations in transcriptional activity of the different genomic domains influence the transgene expression levels. The lower levels of variations of *bar* transcripts among the transgenic plants containing the 35SAMVL*bar* construct could possibly be attributed to the presence of the leader sequence in 5′-untranslated regions (5′-UTR) of the *bar* transcript. However, there are no earlier reports, to the best of our knowledge, on the role of leader sequences in stabilizing transgene expression variations. Such lowering of transgene expression variability has been reported in studies where the transgenes have been flanked by matrix-associated regions (MARs) from chicken lysozyme[30] or scaffold attachment regions (SAR) from plants[31].

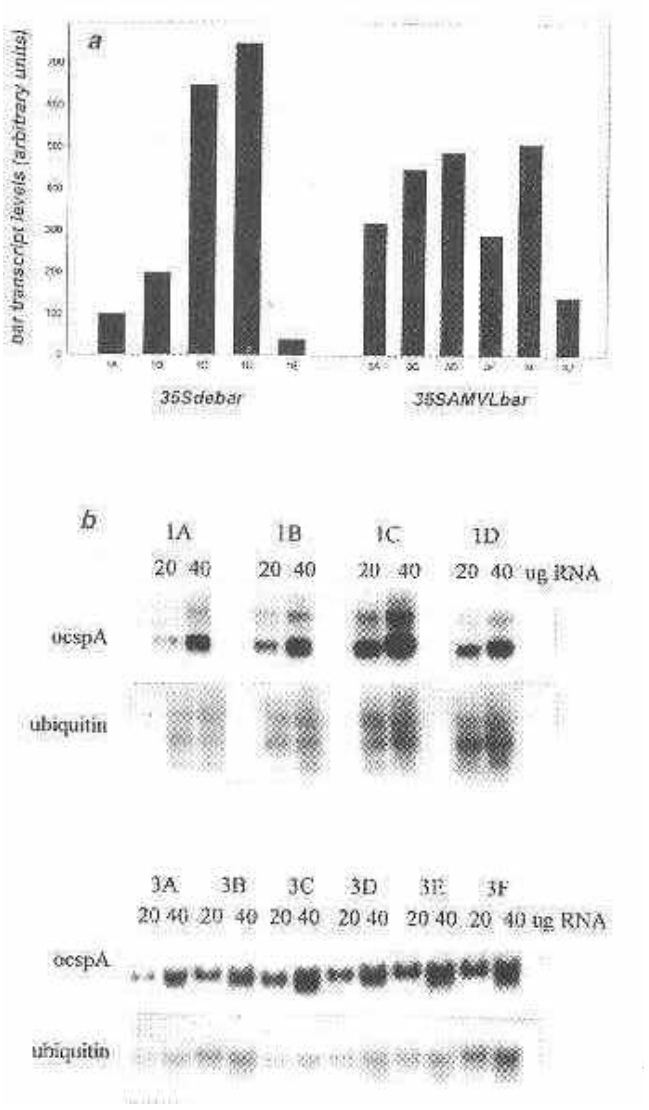

Figure 3. ***a***, Levels of *bar* transcripts observed in different single copy transgenic plants with 35Sde*bar* and 35SAMVL*bar* gene constructs. The transcript levels in different plants have been represented in comparison to values for plant 1A taken to be 100 arbitrary units; ***b***, Representative Northern hybridization patterns following probing of total RNA from different transgenic plants with ocspA fragment to identify *bar* transcripts and with ubiquitin gene probe to normalize loading differences. The signals quantitated through phosphorimager were used to calculate the transcript levels of *bar* in each plant.

Plant viral leader sequences such as TMVL from tobacco mosaic virus RNA[32], AMVL from alfalfa mosaic virus RNA[19] (used in this study), STNVL from satellite tomato necrosis virus RNA[33] which are a part of the 5′-UTR of mRNA have been shown to enhance translational efficiency of transgenes. It has also been demonstrated that enhanced translation protects the transcripts from rapid degradation, thereby indicating the role of leader sequences in mRNA stability.

PAT activity in single copy bar transgenic lines

The levels of bar protein in each of these lines were analysed ^{14}C-Acetyl-PPT assay of the PAT enzyme which is encoded by the *bar* gene. PAT assay was carried out following the protocol of De Block *et al.*[10] using varying concentrations of total protein extracts from different transgenic lines. The PAT activities observed in the different lines are represented in Figure 4 *a* and a representative chromatogram is shown in Figure 4 *b*.

The 35Sde*bar* transgenics showed higher PAT activity when compared to that of the 35SAMVL*bar* transgenics. Percentage conversion per µg of protein varied from 6 to 22% in the case of 35Sde*bar* transgenics with a mean of approximately 13% while conversion observed in 35SAMVL*bar* lines was in the range of 0.8 to 3.5% with a mean of 1.7%. Transformation frequencies observed with the 35Sde*bar* construct and 35SAMVL*bar* (Table 1) also reflect the overall differences in PAT activity observed in lines with the two different constructs. It could be argued that constructs driving higher levels of PAT enzyme (as in the case of 35Sde*bar*) would give better protection and therefore one would be able to select for more number of transformants when compared to constructs driving lower expression levels, viz. 35SAMVL*bar*.

The low PAT activity observed in the 35SAMVL*bar* lines is however, not due to lower levels of transcripts in these lines (Figure 3). It was observed in this study that transformation frequencies with 35S*bar* construct were extremely low (Table 1). The transformation frequency doubled with 35SAMVL*bar* construct, where an AMVL leader sequence

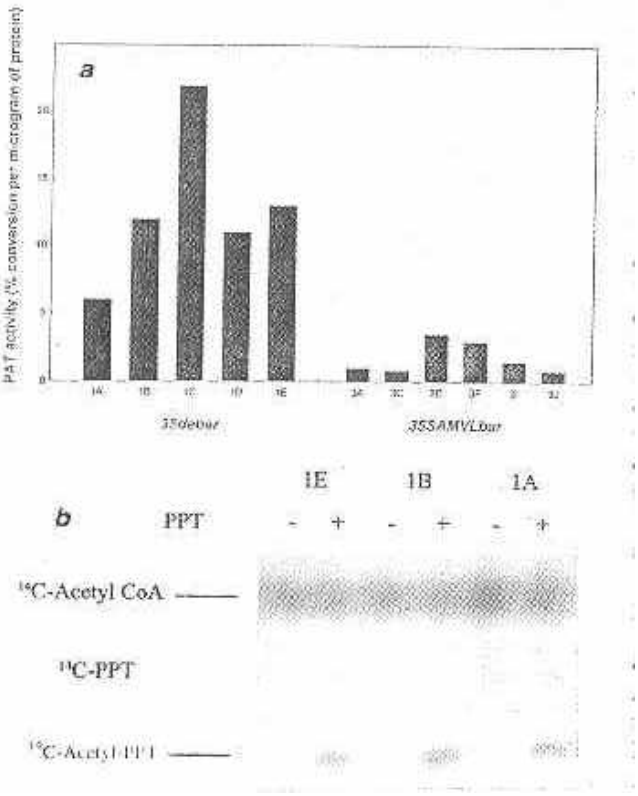

Figure 4. ***a***, PAT activity observed in crude protein extracts from leaves of 35Sde*bar* and 35SAMVL*bar* transgenic plants. PAT activity has been expressed as percentage transfer of ^{14}C from ^{14}C-acetyl CoA to PPT; ***b***, Detection of PAT activity for some of the 35Sde*bar* plants using 1.3 µg of protein extracts by TLC. The extracts were prepared from leaf tissue and different concentrations of protein were used for the reaction. An aliquot was spotted on a silica gel TLC plate for ascendent chromatogarphy. ^{14}C substrate (^{14}C-AcetylCoA) and the reaction product (^{14}C-Acetyl-PPT) as visualized by autoradiography are

was cloned in the 5′-UTR of the *bar* gene in the 35S*bar* construct. As already discussed, such leader sequences lead to increased translational efficiency of mRNA. The transcript levels in the 35S*bar* (data not shown) and 35SAMVL*bar* lines were similar but no PAT activity could be recorded in the former lines. It would thus mean that the inclusion of the AMV leader sequence in the 5′-UTR of the *bar* mRNA led to increase in its translational efficiency but not as high PAT levels as observed in the case of 35Sde*bar*. It should be mentioned here that due to the cloning strategies adopted there are minor sequence differences in the 5′-untranslated region of the *bar* transcript, transcribed from each of these three constructs.

The PAT activity levels observed in individual transgenics is enough to give tolerance at the tissue culture level and thus these constructs can be used as selectable markers in transformation experiments. However, these lines need to be tested for resistance by spraying different concentrations of the herbicide at the field level. It has been reported[10] that transgenic lines with as low as 0.001% PAT protein of the total protein have sufficient resistance against PPT at a concentration of 8 L/ha Basta (200 g/l PPT). However, these levels may not be sufficient in every crop. The levels of expression required for full protection may also vary under different agroclimatic conditions. Transgenic plants which showed variable expression levels have been selfed to generate homozygous lines for field-testing and back-crossed to study expression levels in the BC2 generation.

Selected homozygous lines which would show full yield potential after herbicide treatment in the field could be further used for agronomic trials under no-till or low-till cultivation conditions in the mustard growing areas. James[2] has listed six major advantages that have occurred from the use of herbicide-resistant transgenics in the North American continent. For cropping systems used in India the most interesting use of herbicide resistant transgenics will be to develop models for low-till or no-till agriculture for moisture and soil conservation. Trans-genics produced in this study would allow testing of such models in the rain-fed areas. In no-till agriculture seeds are sown in unploughed soil to conserve water and later the weeds are removed by spraying herbicides. The crop planted being resistant to herbicide survives. This technology if applicable may allow intensification of agri-culture and more importantly may help in the conservation of moisture and soil fertility in rain-fed areas.

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