

The Role of Altered Glutamine Synthetase in Conferring Resistance to Glufosinate in Mungbean Cell Selections

T. Pornprom^{1*}, A. Pengnual², N. Udomprasert¹ and O. Chatchawankanphanich³

¹Department of Agronomy, Faculty of Agriculture

Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand

²Center of Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus
Nakhon Pathom 73140, Thailand

³Plant Research Group, BIOTEC Central Research Unit

National Center for Genetic Engineering and Biotechnology

Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand

*Corresponding author. Email: agrtpp@ku.ac.th

Abstract

Selection of glufosinate resistant mungbean cell line (*Vigna radiata* L. Wilczek cv. Shaanxi) was performed through in vitro mutant selection to generate herbicide-resistant crops. Cell suspension was cultured on MS medium supplemented with 2,4-D 3 mL L⁻¹, NAA 1.5 mg L⁻¹, BA 0.5 mg L⁻¹ and coconut water 10% L⁻¹ (pH 5.6). In vitro selection from these cell suspension cultures resulted in the identification of a mungbean cell line resistant to glufosinate. Based on the I₅₀ values, the resistance index of the resistant cells was 333-fold more than the normal cells. It is referred to as 10⁻⁶ M glufosinate-resistant mungbean cell line. Determination of glutamine synthetase (GS) activity of resistant cells showed an approximate 2.65-2.95 fold increase compared to normal cells, whereas ammonium accumulation in resistant cells showed a 1.25-1.35 fold drop compared to normal cells. In addition, the GS gene fragments in normal and resistant cells were amplified, cloned and sequenced. Sixteen different amino acid substitutions were observed between the GS gene from resistant and normal cells. Based on these findings, alterations to the GS gene in resistant cells might correlate with target site-based mechanisms, resulting in increased activities of GS enzyme less affected by glufosinate.

Keywords: amino acid substitutions, ammonium accumulation, glufosinate-resistant, mungbean cell line, glutamine synthetase (GS), GS gene, point mutation

Introduction

Glufosinate is a relatively nonselective post-emergence (POST) herbicide. It controls a wide range of annual and perennial grass and broadleaf weeds (William, 1994). The use of this herbicide, combined with a unique mode of action, would give the farming community a new option in weed control. The herbicide inhibits the activity of an enzyme glutamine synthetase (GS; EC 6.3.1.2), resulting in a rapid accumulation of ammonium, a

deficiency in several amino acids, and an inhibition of photosynthesis within the plant (Wild and Wendler, 1990; William, 1994; Donn and Köcher, 2002). This inhibitory process prevents the detoxification of ammonium released by metabolic processes in photorespiration, catabolic metabolism of amino acids and nucleic acids and nitrite reduction, resulting in the accumulation of toxic ammonium within the plant cells, membrane breakdown (lipid peroxidation) and finally in the death of the plant cells (Sankura et al., 1998).

The use of herbicides to control weeds allows growers to practice more efficient crop management, together with improving yields. Several classes of herbicides can be effectively used for broad-spectrum weed control, but these are either non-selective, killing also the crop plants, or they significantly injure some crops at the application rates required. The development of herbicide-tolerant cultivars is one way in which crops can be protected from herbicide damage. Three general methods have been used to generate herbicide-resistant crops: germplasm screening (mutant isolation by conventional breeding), *in vitro* selection (mutant isolation in cell cultures) and direct insertion of a herbicide-resistant gene using the genetic engineering approach (creating mutants) (Hinchee et al., 1993).

Various strategies have been applied in order to produce glufosinate-resistant crop plants (Dröge et al., 1992; Mullner et al., 1993; Hart et al., 1994; Hoerlein, 1994). Cell selection methods are very useful to increase herbicide-resistance in crop plants, and are essential if genetic engineering methods are to be attempted (Gressel, 1987). To date, cell suspension cultures have been frequently used to investigate herbicide target sites and their involvement in herbicide actions relating to resistance mechanisms. In addition, the target genes conferring resistance have been isolated and correlated with the biochemical mechanisms and/or molecular bases of herbicide resistance. In this study, a glufosinate-resistant mungbean cell line was produced through tissue culture using stepwise selection methods. The availability of glufosinate-resistant mungbean cell line should be of great agronomic interest and should expand the use of the herbicide, which is now limited due to its nonselective mode of action. Therefore, the objective of this study was to further describe a different biochemical and molecular basis for the resistance mechanism of the mungbean resistant cells to glufosinate. The biochemical basis of resistance in the resistant cells was investigated by *in vitro* GS activity assays and ammonium accumulation test, while the molecular basis of resistance was determined through the identification of the GS gene mutant in normal and resistant cells of mungbean.

Materials and Methods

Selection of Glufosinate-Resistant Mungbean Cell Line

Seeds of mungbean cv. Shaanxi were plated for germination on hormone-free solid MS medium (Murashige and Skoog, 1962). After culturing for 7 days, the hypocotyls were cut and transferred to solid MS medium, supplemented with NAA 1.50 mg L⁻¹ and BA 0.50 mg L⁻¹. After 1 month, friable callus was selected from compact organogenetic calli and used for induced cell suspension. Friable embryogenic calli were transferred to a 250 mL Erlenmeyer flask containing 45 mL of liquid modified MS medium consisting of 2,4-D 3 mg L⁻¹, NAA 1.5 mg L⁻¹, BA 0.5 mg L⁻¹ and coconut water 10% L⁻¹ (pH 5.6). The flasks were placed on a gyrating shaker at 120 rpm and 28°C. The growth rate was examined by measuring packed cell volume (PCV) to find the appropriate rate for sub-culturing cells.

Calli were sub-cultured at 10 days interval by transferring to a fresh medium with an initial cell density of approximately 1 mL of PCV in 45 ml of growth medium. The mungbean suspension cells were treated with 10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶ and 10⁻⁵ M glufosinate, respectively. The normal cells were sub-cultured using non-treated herbicide for control. The growth rate was determined by measuring the PCV at 3, 5, 7, 10 and 14 days after treatment (DAT) to evaluate the effect of herbicide on the growth of mungbean cells. The suspended cells were treated at the lowest concentration of 10⁻⁸ M glufosinate. Cells were sub-cultured at 10 days interval by transferring the cell suspensions to fresh medium with the same concentration of the herbicide for the entire experiment. When the growth rate reached the same level as that of the normal cells (without glufosinate), the cells were transferred into a medium containing a higher concentration of 10⁻⁷ M glufosinate. Sub-culturing was repeated until highly resistant cells were obtained.

Glutamine Synthetase Activity

All experiments for in vitro GS activity assay were carried out at 4°C. The enzyme extraction and assays were performed according to the method of O'Neal and Joy (1973) and Forlani (2000). Each 2 g sample of mungbean cells was homogenized with liquid nitrogen and added to an extraction buffer (0.05 M Tris HCl buffer (pH 7.2), 0.01% mercaptoethanol and 0.5 mM Na₂EDTA). The mixture was centrifuged at 15,000 *xg* for 15 min and the resulting supernatant was immediately used as crude extract for the enzyme assay.

For in vitro GS activity assays, the crude extract (0.4 mL) was added to the reaction mixture (1 mL) containing 80 mM sodium glutamate, 16 mM ATP, 50 mM MgSO₄, and 50 mM imidazole-HCl at pH 7.4 then incubated at 25°C for 5 min. The reaction was initiated by adding 0.1 mL hydroxylamine solution (1 M NH₂OH: 1M NaOH: H₂O; 1: 1: 2, v/v/v) and technical-grade glufosinate at a concentration of 10⁻⁶ M. After incubation at 25°C for 15 min, the reaction was stopped by adding 0.4 mL ferric chloride solution (8 g FeCl₃ and 10 g trichloroacetic acid dissolved into 250 ml of 0.5 M HCl). Following centrifugation of the sample at 3,000 *xg* for 10 min, absorbance of the supernatant was measured at 540 nm. Absorbance was converted to units of GS activity using a standard curve produced from known concentrations of L-glutamic acid- γ -hydroxamate. The protein content was determined according to the methods of Bradford (1976) using bovine serum albumin as standard. The protein and enzyme activity were calculated by fitting data to linear regression model: $Y = a + bX$, where X is the explanatory variable (the dose of GS), Y , the dependent variable (absorbance value), b , the slope and a , the intercept. GS activity was expressed in $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein.

Ammonium Accumulation

The ammonium accumulation was assayed according to the modified procedures of Desmaison et al. (1984). Cell samples (2 g fresh weight) were homogenized with liquid nitrogen, extracted with 10 mM HCl and 0.18 g of sulfosalicylic acid, kept at room temperature for 15 min, and then centrifuged at 3,000 rpm for another 15 min. To determine ammonium accumulation, the supernatant (200 μL) was diluted with 800 μL of distilled water. The

diluted supernatant (20 μL) was added with 2-5 mL Reagent A (1 g phenol, 5 mg sodium nitroprusside and 100 mL distilled water) and 2.5 mL Reagent B (0.5 g NaOH, 0.84 mL NaOCl and 100 mL distilled water). The reaction mixture was incubated at 37°C for 20 min and the absorbance was measured at 625 nm using a spectrophotometer. The standard curve was obtained using NH₄Cl at concentrations ranging from 0.5 to 4 μL . Ammonium accumulation was expressed in mmol g^{-1} fresh weight.

Extraction of RNA and

Identification of Partial GS Gene Mutant

Total RNA was isolated from resistant and normal cells as described by the manufacturer of the Puregene[®] Kit (Gentra system, USA). Four primers were used to amplify the coding part of the GS sequence. Primer GS1-F: 5'-CCA AAG TGA AAC TAI GAI GGC TCY AGY AC-3' and primer GS6-R: 5'-TCC CAC TGA CCA GGC ATI ACY TC-3' were used to produce the amplified 447-bp 5' end fragment of the GS gene as reported by Perez-Vicente et al. (1996). Primer GS363-F: 5'-CAR GGN CCN TAY TAY TGY-3' and primer GS1038-R: 5'-AAC CAC GTA TGG GTC CAT-3' was used to amplify 486-bp 3' end fragment of the GS gene. The corresponding regions of the *V. radiata* GS genes were synthesized from 3 μL of total RNA by using the one-step RT-PCR (QIAGEN, Germany) with primer GS1/GS6 and primer GS363/GS1038. The conditions for RT-PCR consisted of reverse transcription for 30 min at 50°C, initial PCR activation step for 15 min at 95°C, followed by 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and final extension at 72°C for 1 min. The amplified products were sequenced after cloning into the pGEM[®]-T easy vector system (Promega, USA) following the manufacturer's instructions. Nucleotide sequences were compared with other known GS gene by the Megalign program in DNASTAR lasergene software (DNASTAR, USA).

Results and Discussion

Selection of Glufosinate-Resistant Mungbean Cell Line

The effect of glufosinate on the growth of normal mungbean cells is presented in Figure 1.

The results showed that there were significant differences in the effect of glufosinate concentrations on the growth of normal cells. The inhibition of growth escalated as glufosinate concentration was increased. Growth was strongly inhibited at 10^{-5} M glufosinate.

The selection of resistant cells was initiated by stepwise selection from normal cells. At first, the normal cells were cultured in liquid MS medium containing 10^{-8} M glufosinate. The growth rate was found to be low at the early stage of the selection but gently reached the same rate as the normal cells after 40 days (data not shown). In the next step, the cell suspensions were transferred to 10^{-7} M glufosinate. Growth rates of the cells were the same as in the first step after 60 days. The growth rate had recovered to the same level as that of the normal cells after 70 days. The selection of the glufosinate-resistant mungbean cell line was terminated at 10^{-6} M glufosinate, because cells died in higher concentrations. The mungbean cell line resistant to 10^{-6} M glufosinate was obtained after 170 days of selection. Based on the I_{50} values, fifty percent growth inhibition of normal cells occurred at 7.5×10^{-8} M and were completely dead at 2.5×10^{-5} M (Figure 2). At this concentration, on the other hand, resistant cells still grew successfully and gave a resistance index of 333-fold greater than the normal cells.

The successful selection of glufosinate resistant mungbean cell line was an encouraging result as it may be useful in crop improvement. Previously, the selection of glufosinate resistant cells by the stepwise selection of herbicide was successful for soybean (Pornprom et al., 2000). Additionally, there have been many reports on herbicide resistant crops from tissue culture selection. Bae et al. (2002) selected rice (*Oryza sativa* L. cv. Ilpumbyeo) cells resistant to cyhalofop-butyl. Soybean cells were screened for Protox-inhibiting herbicides resistance (Pornprom et al., 1994; Warabi et al., 2001). Imidazolinone-resistant sugarbeet (*Beta vulgaris*) cells were obtained by somatic cell selections (Wright et al., 1998). However, none of the previous reports studied the biochemical mechanisms at the target sites of glufosinate action and the molecular basis of resistance of mungbean cells. The results from this study could subsequently be attributed to the mechanism of resistance to glufosinate in mungbean cell selections.

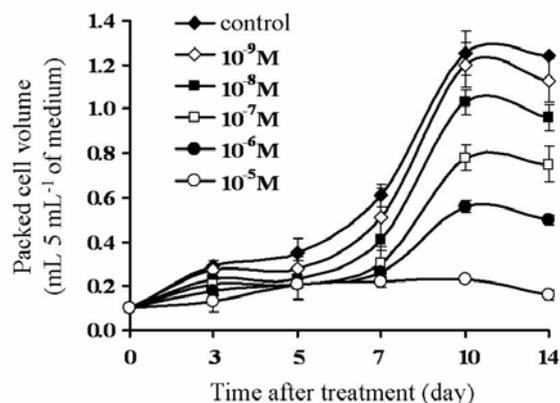


Figure 1 Effect of glufosinate on the growth of normal mungbean cells 3-14 days after treatment with 0, 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} and 10^{-5} M. Points are the average of three replicates \pm standard errors.

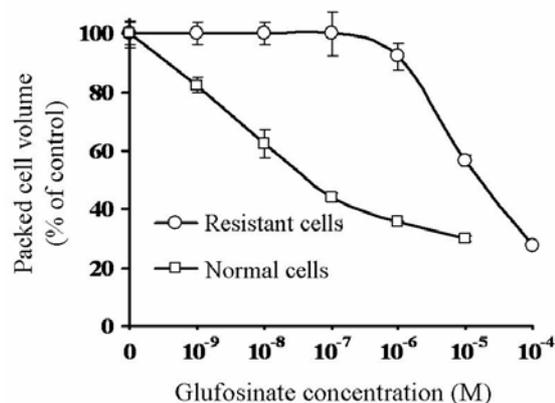


Figure 2 Growth response of normal and resistant cells to glufosinate 10 days after treatment. Points are the average of three replicates \pm standard errors.

Glutamine Synthetase Activity

GS activity was assayed *in vitro* in the presence of glufosinate to investigate whether the mechanism of resistance was a target site alteration in resistant mungbean cells. The specific activity of GS in normal cells without herbicide treatment (N) ranged from 39.36 to 40.60 $\mu\text{mole min}^{-1} \text{mg}^{-1}$ protein, whereas the specific activity of GS in resistant cells without herbicide treatment (R) ranged from 38.48 to 40.65 $\mu\text{mole min}^{-1} \text{mg}^{-1}$ protein. The results showed that without herbicide treatment, the level of GS specific activity in both N and R cells was not significantly different. This result indicates that resistance to herbicide in

resistant cells was not due to enhanced GS activity. When both types of cells were treated with 10^{-6} M glufosinate, however, there was a significantly higher level of GS activity in herbicide-treated resistant cells (RT) compared with herbicide-treated normal cells (NT) (Figure 3). GS activity was reduced in normal cells but increased in the resistant cells. Based on the R/N ratio, GS activity of the resistant cells was approximately 2.65-2.95 times more resistant to glufosinate than the normal cells. Hence, resistance to glufosinate in the resistant mungbean cells is due to the activity of a herbicide-resistant GS enzyme. The results suggest that resistance involved a reduction in the sensitivity of the GS of the resistant mungbean cells to glufosinate. This confirmed the suspected resistance in the cell cultures.

The present study of the mechanism responsible for resistance in the mungbean cells investigated indicated that there was closed association between the concentration-response at the cell level and GS sensitivity to glufosinate. The cells with target site-based resistance have GS that is less affected by glufosinate than GS enzyme from normal cells. These results were consistent with earlier reports on the mechanisms of GS-inhibitor resistance in several plants (Krieg et al., 1990; Tsai et al., 2006). These

data suggest that the higher activity of GS in resistant cells was due to less sensitivity to glufosinate conferring the mechanism of resistance. This study clearly showed that herbicide resistance can be due to target site-based mechanisms.

Ammonium Accumulation

The accumulation of ammonium in the normal cells without herbicide treatment (N) was lower than in the resistant cells without herbicide treatment (R) (Figure 4). When cells were treated with 10^{-6} M glufosinate, the ammonium accumulation in the herbicide-treated normal cells (NT) rapidly increased and was found to be higher than in the herbicide-treated resistant cells (RT), particularly at 7 to 14 DAT. As regards the ammonium accumulation, a biochemical marker of GS inhibition by glufosinate, the levels of accumulation in the resistant cells were approximately 1.25-1.35 fold lower than in the normal cells. Comparable results were also observed in glufosinate-resistant soybean cell lines (Pornprom et al., 2000). This suggests that the resistant cells had some mechanism to prevent the accumulation of ammonium caused by glufosinate, thereby allowing the mungbean cells to survive the glufosinate treatment.

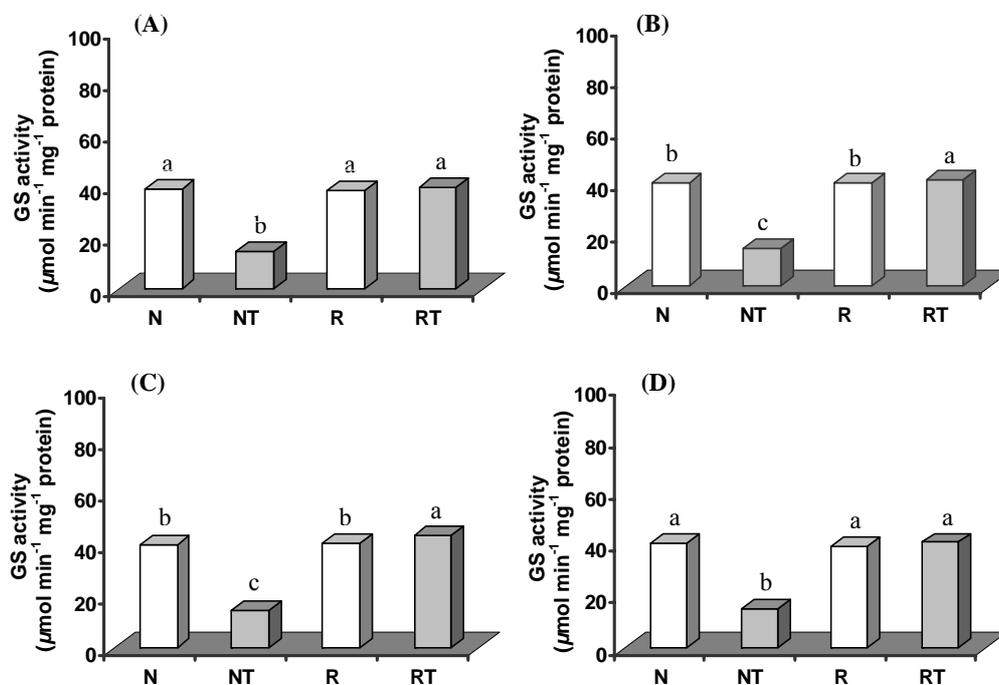


Figure 3 GS activity in normal cells without herbicide (N), normal cells treated with 10^{-6} M glufosinate (NT), resistant cells without herbicide (R) and resistant cells treated with 10^{-6} M glufosinate (RT) at 3 (A), 5 (B), 7 (C) and 10 (D) days after treatment of herbicide. Bars with the same letter within cell types are not significantly different at $P = 0.01$.

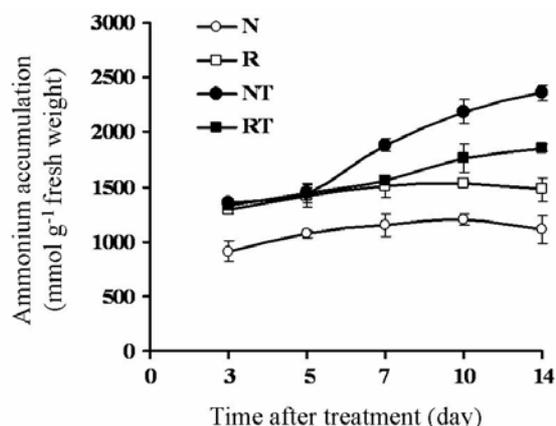


Figure 4 Ammonium accumulation in the normal cells without herbicide treatment (○), normal cells treated with 10^{-6} M glufosinate (●), resistant cells without herbicide treatment (□), and resistant cells treated with 10^{-6} M glufosinate (■) at 3, 5, 7, 10 and 14 days after treatment. Points are the average of three replicates \pm standard errors.

This inhibition of GS activity implied that less glutamine was formed and that glutamate and ammonium would accumulate in the plants. GS inhibition prevents ammonium ions from being incorporated into amino acids, resulting in the accumulation of ammonium ions to toxic levels within the plant cells and leading to membrane breakdown and cell death (Sankura et al., 1998). The GS activity in resistant cells was altered which might be due to a change of the sensitivity of GS enzyme to glufosinate. This result correlated with that from the GS activity assay experiment and indicated that resistance involved a reduction in the sensitivity of the GS of the resistant cells to glufosinate. A similar relationship between glufosinate resistance and ammonium accumulation has been reported in alfalfa (*Medicago sativa* L.) (Krieg et al., 1990), soybean (Pornprom et al., 2000), and rice (Tsai et al., 2006). From this study, it is clear that higher GS activity and lower ammonium accumulation in the resistant cells could confer the biochemical mechanisms of glufosinate resistance. This can be used as an indicator to select for glufosinate-resistant mungbean cell line.

Identification of Partial GS Gene Mutant

The DNA fragment with the expected size of 447 bp was produced by primer GS1-F and GS6-R, whereas primer 363-F and 1038-R amplified the 570 bp fragment of GS gene (Figure 5). All sequences were deposited in the GenBank database (GenBank Accession No. AY918119 and AY918120 for resistant and normal cells, respectively). A sequence of 281 amino acids was deduced from each nucleotide sequence. The deduced GS amino acid sequence of resistant cells was 94.3% identical to that of the normal cells. Sixteen different amino acid substitutions were observed between the GS gene from resistant and normal cells (Figure 6 and Table 1). It has been reported that herbicide resistance was due to structural alterations in the target protein. Sulfonylurea resistance in *Brassica napus* resulted from an alteration of the acetohydroxy acid synthase which made it less sensitive to the herbicide (Hattori et al., 1995). Change in sensitivity to ALS-inhibiting herbicides has been related to mutations at the binding site of the herbicides on the ALS enzyme (Osuna et al., 2003). Furthermore, different amino acid substitutions at the same mutation site may confer different levels of resistance to one or a group of specific herbicides (Dewaele et al., 1996). However, no comprehensive study has been published to show that glufosinate resistance is due to mutations of the GS gene. These results suggest that the amino acid substitutions in GS gene might be correlated with GS protein as enzyme activity. However, the functional protein should be further studied to elucidate the affect of this phenomenon in resistant cells.

In the present study, the glufosinate-resistant mungbean cell line was investigated through in vitro mutant selection to generate herbicide-resistant crops. Even though it was possible to select a mungbean cell line with increased resistance for glufosinate which was 333-fold higher than the resistance level of the original cell line, all attempts to regenerate plants from the mutant cell line failed. However, regeneration of resistant cell lines is being undertaken. The biochemical and molecular basis of resistance to glufosinate in mungbean were evaluated at the

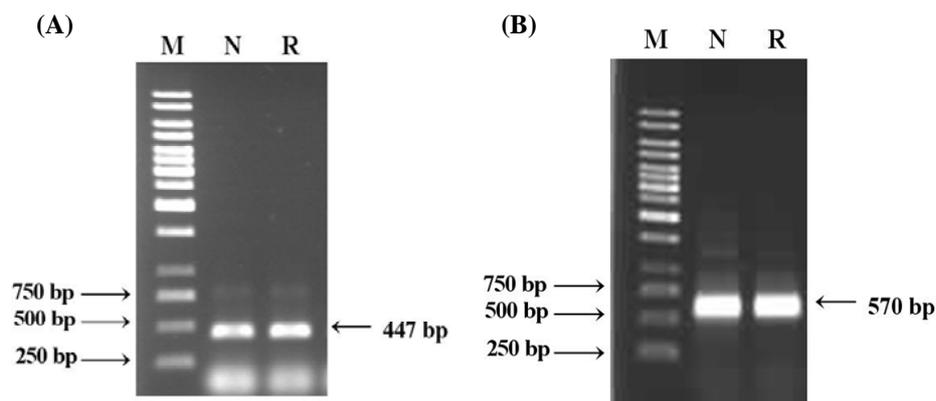


Figure 5 DNA fragment of partial GS gene amplified 447 bp by primer GS1 and GS6 (A) and 570 bp by primer GS 363 and GS 1038 (B) from normal and resistant mungbean cells.

Table 1 Nucleotide substitutions and predicted amino acid changes in normal and resistant mungbean cells conferring resistance to glufosinate.

Nucleotide position ^{1/}	Nucleotide substitution		Amino acid substitution	
	Normal cell	Resistant cell	Normal cell	Resistant cell
14	GGT	GAT	Gly	Asp
30	GAA	GAT	Gly	Asp
46	ATA	TTA	Ile	Leu
113	GCT	GTT	Ala	Val
136	ATT	CTT	Ile	Leu
140-141	CCC	CAA	Pro	Gln
154, 156	CAC	TAT	His	Tyr
157, 159	AAC	GAT	Asn	Asp
271	GTT	CTT	Val	Leu
292-293	CAG	AGG	Gln	Arg
296-297	GGA	GAT	Gly	Asp
310	GGT	AGT	Gly	Ser
325-327	AAG	CGT	Lys	Arg
328, 330	GCT	TCA	Ala	Ser
346, 347	GTT	TCT	Val	Ser
374	TAT	TTT	Tyr	Phe

^{1/} The numbers refer to partial GS coding sequence of *Vigna radiata* L. (Genbank Accession No. AY918119).

cellular level. From the data available, it can be concluded that glufosinate, as a consequence of GS inhibition, leads to plant death by multiple interference with plant metabolism. A combination of resistance mechanisms could lead to a very high level of resistance to the herbicide. From the

results, the alteration in the GS gene in resistant cells may be correlated with target site-based mechanisms, resulting in an increased activity of GS enzyme that is less affected by glufosinate. Furthermore, this inhibition of GS activity implied that less glutamine was formed, and that glutamate

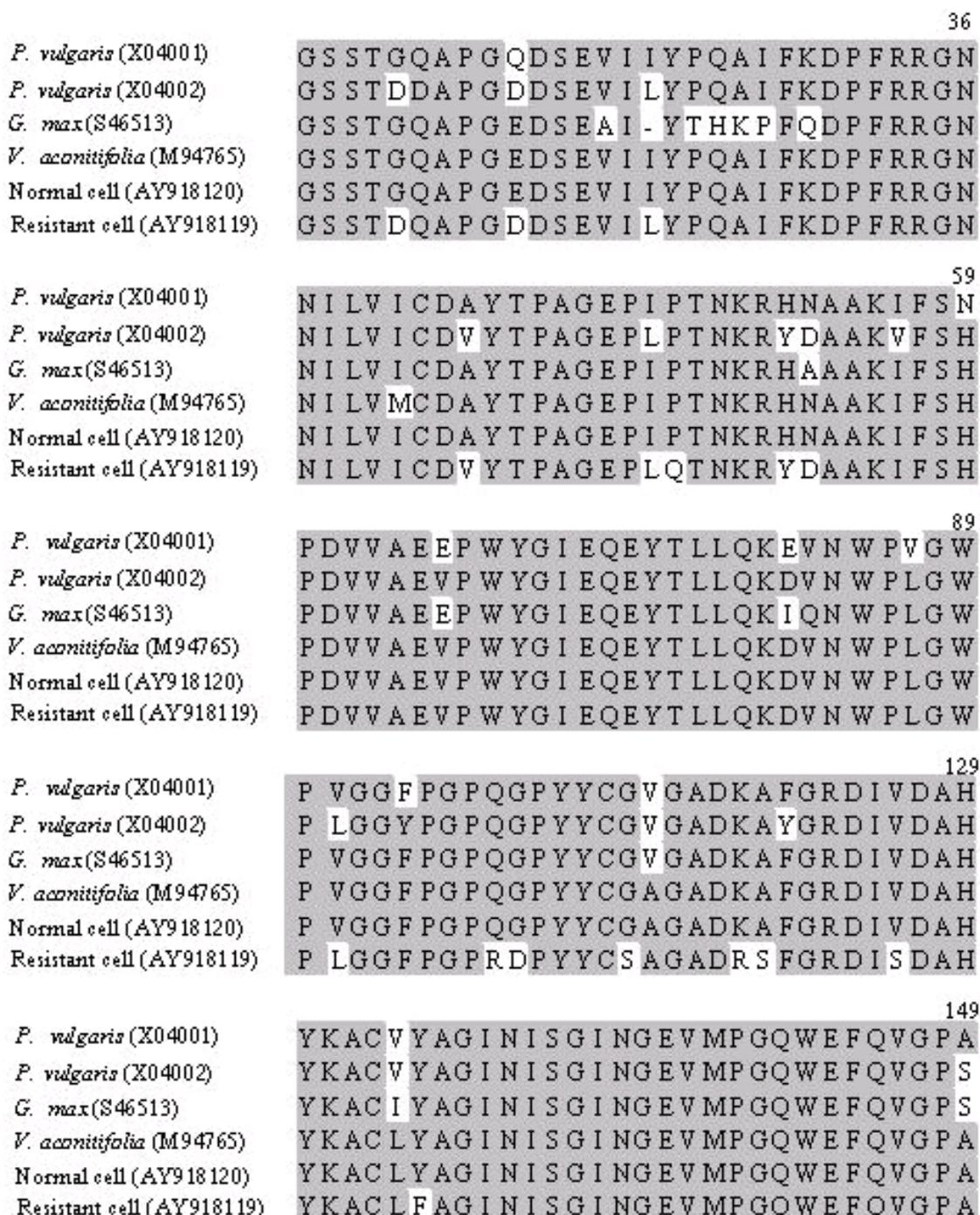


Figure 6 Alignment of the deduced amino acid sequences of partial GS gene in resistant cells (AY918119) and normal cells (AY918120) with the GS from GenBank of sub-family Fabaceae. Identical amino acids substituted are shaded with light gray. The deduced GS amino acid sequence of resistant cells was 94.3%, 93.6%, 91.1%, 89.3% and 86.5% that of the normal cells, *V. aconitifolia* (M94765), *P. vulgaris* (X04001), *G. max* (S46513) and *P. vulgaris* (X04002), respectively.

and ammonium would accumulate in the plants. Based on these findings, it was suggested that herbicide resistance may be due not only to target site-based mechanisms but also to a point mutation of the GS gene. Most importantly, point mutation

of a plant GS gene may lead to a mutant enzyme with lowered binding affinity for glufosinate. However, such mutants may not be viable because of the reduced binding affinity for glutamate and hence its reduced enzymatic activity.

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