International Journal of Basic and Applied Biology
Print ISSN: 2394-5820, Online ISSN: 2349-2539, Volume 2, Number 3; October-December, 2014 pp. 173-175

http://www.krishisanskriti.org/ijbab.html

Glyphosate Detoxification Ability of Crude Enzyme Extracts from Transgenic Tobacco Plants Expressing Bacterial Genes

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Abstract: *Glyphosate is a broad spectrum systemic herbicide with low animal toxicity and no residue in soil. Glyphosate targets EPSPS in aromatic amino acid biosynthesis pathway. A modified CP4-EPSPS from Agrobacterium tumifaciens is not affected by glyphosate and transgenic tobacco and rice expressing CP4-EPSPS are tolerant to glyphosate. igrA and Glycine oxidase detoxifies the glyphosate by cleaving C-P and C-N bond respectively. Transgenics expressing both CP4-EPSPS and detoxifying enzymes improves tolerance and reduces the level of glyphosate. With this hypothesis, glyphosate tolerant lines was developed in tobacco. In present study, the T_2 generation tobacco lines developed for glyphosate tolerance by expressing CP4-EPSPS, Glycine oxidase, igrA alone and in combination (Double and multiple) were assessed for their relative tolerance based on physiological and molecular analysis.*

1. INTRODUCTION

The herbicide glyphosate (*N*-(phosphonomethyl) glycine) is a 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase inhibitor in plants. The enzyme participates in biosynthesis of aromatic amino acids phenylalanine, tyrosine and tryptophan [1]. Glyphosate acts as a competitive inhibitor for phosphoenolpyruvate and is used as a broad-spectrum systemic herbicide. Development of glyphosate-tolerant crops allows preferential and more effective use of glyphosate resulting in fewer or more environmentally benign chemical inputs. Glyphosate-resistant crop varieties were developed by inserting glyphosate resistant clone *CP4-EPSPS* into plants [2, 3]. This transgene allows the shikimate pathway to function in presence of glyphosate, thus allowing plants to survive after glyphosate application. In addition to introducing *CP4-EPSPS* gene, introducing specific genes to cleave the glyphosate molecule in plants gives additional advantages to by having additional potential to reduce the glyphosate injury as well as removing the toxic molecule of glyphosate from the tolerant crop varieties.

Two broad strategies were adopted to develop glyphosate tolerant crop varieties, In the first strategy which is mostly adopted in all "Round up" ready crops the target enzyme of glyphosate *EPSPS* was made to over produce in an insensitive form, so that there will be enomorously more amount of glyphosate insensitive *EPSPS*, resulting in continuous synthesis of aromatic amino acid in plants even after application of herbicide glyphosate.

In the second strategy the herbicide glyphosate entered into the plant system is metabolized immediately by glyphosate detoxifying enzymes which catabalizes herbicide glyphosate into non toxic metabolites. To achieve the second strategy two genes from soil bacteria which are capable of producing enzymes which cleave the glyphosate to non toxic forms have been identified and introduced into plants.

The two genes *irgA* cloned from *Pseudomonas* strain PG 2982 and *GOX* from *Bacillus subtilis* were shown to detoxify glyphosate by breaking glyphosate molecule at C-P bond and C-N bond respectively [4, 5]. It is hypothized that developing transgenic lines over producing *EPSPS* and also capable of detoxifying glyphosate in the plant will be more useful in developing varieties for glyphosate tolerance. In order to study the detoxifying ability these genes, tobacco plants expressing *igrA*, glycine oxidase (*GOX*) from and *CP-4 EPSPS* genes in different combinations (single, double and multiple) were developed. The detoxification ability of the transgenic tobacco plants were studied by response of the cucumber seedlings grown on reaction product of glyphosate treated with crude enzyme extracts of the transgenic tobacco leaves for varied reaction time.

2. MATERIALS AND METHOD

2.1 Plant material

In the present study the putative T2 transgenic tobacco seed material was forwarded to next generation during which

physiological and molecular studies were made. The transgenic lines comprises of 1.Multiple Construct **(M)**: Expressing EPSPs, igrA and GOX. 2. Double Construct: Expressing igrA and GOX **(IG**) and igrA and EPSPs **(IE).** 3. Single construct: expressing GOX **(G**), IgrA **(I)** and EPSPs (**EP**) in single.

2.2 Extraction of the crude enzyme from the T2 transgenic tobacco plants

Transgenic tobacco lines expressing *EPSPS, igrA*, and *GOX* either in single or in combinations were used for the extraction of the crude enzyme from leaf tissue collected from 58 days tobacco plants. Crude enzyme was extracted with 1 X PBS (phosphate buffer saline) of PH 7.4 on ice cold condition by grinding with pestle and mortar and centrifuge at 16,000 rpm for 1 hr and supernatant (crude enzyme) collected was used for the enzyme assay.

2.3 Cucumber seedling root growth inhibition assay

Crude enzyme extract was prepared from the leaves of 58 day old transgenic tobacco plants and crude extract was used to react with a known concentration of glyphosate. Reaction is stopped at different interval of time and the reaction product was used to incubate germinated cucumber seedlings to study the relative influence of different transgenic lines in detoxifying the glyphosate. The extent root growth of cucumber seedlings indicates the ability of different transgenic lines in glyphosate degradation. To 200 µL of crude enzyme aliquot, 25 µL of 100 ppm glyphosate and 500 µL of PBS Buffer was added and incubated for different intervals viz 0, 10, 30, 60, 120 and 180 min and reaction is stopped by adding 100 µl of absolute alcohol to the reaction mix and the total volume was made to 6 ml with buffer. One day old pre germinated cucumber seedlings were incubated in petridish over filter paper soaked with 6 ml of reaction mixture. The root growth was measured after 3 days of reaction mixture treatment. Based on the ability of the enzyme and reaction time given, glyphosate will be degraded to different extent resulted in differential influence on root growth of cucumber seedlings.

3. RESULT AND DISCUSSION

The gene product *GOX* and *igrA* are known to degrade the glyphosate into AMPA and sarcosine respectively, the degraded products were known to have less herbicidal effect [6,7]. In order to study the efficacy of protein extracted from transgenic tobacco expressing *EPSPS*, *GOX, igrA* alone or in combination of double and multiple gene construct on detoxifying ability an experiment was conducted in which crude enzyme extract from transgenic lines were allowed to degrade a known concentration of glyphosate.

The protein extracted from *igrA* + *GOX* (IG) had relatively higher glyphosate detoxifying ability by showing higher root growth of 4.0, 8.2, 13.3, 12.3 and 15.0 mm at 10, 30, 60, 120 and 180, minutes of incubation period in glyphosate respectively. Among the transgenics lowest root growth was observed in protein extracted from transgenic expressing *EPSPS* (Table 1, Fig. 1 and 2).

Table 5: Relative efficiency of crude enzyme extract of tobacco transgenic lines in degrading glyphosate

I Treated-	igrA alone treated with glyphosate
G Treated-	GOX alone treated with glyphosate
EP Treated-	EPSPs alone treated with glyphosate
IG Treated-	igrA and GOX treated with glyphosate
M Treated-	igrA, EPSPs and GOX treated with glyphosate
WT Treated-	Wild Type treated with glyphosate
WT-	Wild Type

Lines	Root growth (mm)					
	0 Min	10 Min	30 Min	1 hr	2 hr	3 hr
I	1.0	2.3	7.3	10.0	12.5	13.0
G	1.0	4.0	7.0	10.0	12.4	13.5
EP	1.0	2.0	4.0	4.3	4.0	4.0
IG	1.0	4.0	8.2	13.3	12.3	15.0
M	1.3	3.0	3.3	12.3	12.3	14.2
WT	1.0	1.0	1.0	1.0	1.0	1.0
WT Control	20.0	20.0	20.0	20.0	20.0	20.0
CV (%)	5.80%	15.73%	19.30%	10.30%	8.84%	8.88%
CD@0.05	0.2	0.9	1.6	1.2	1.1	1.1

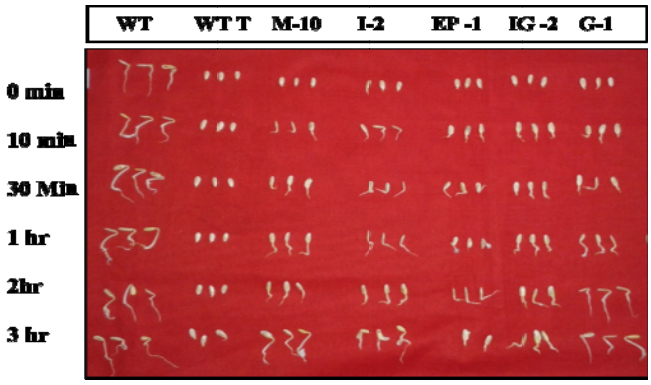

Fig. 1: Variation in response of cucumber seedlings treated with crude protein extracts of transgenic tobacco on glyphosate at different interval of time.

The protein extracted from wild type and incubated with glyphosate showed least growth of root. The data indicates the detoxification mechanism of glyphosate is functioning in transgenic lines expressing *GOX* and *igrA* either in single or in combination of genes. There were several reports indicating the detoxification strategy by the *GOX* gene isolated from *Ochrobactrum anthropi* (formerly *Achromobacter sp.*) Strain LBAA was inserted into the plant. The *GOX* gene encodes

glyphosate oxidoreductase (*GOX*), and the enzyme can degrade glyphosate to glyoxlate and AMPA [8-11]. As a result, *GOX*-catalyzed metabolism of glyphosate reduces the amount of glyphosate that can reach the target enzyme *EPSP synthase*, and thus reduces the possibility of glyphosate injury to the plant.

4. CONCLUSION

The gene product *GOX* and *igrA* are known to degrade the glyphosate into AMPA and sarcosine respectively, the degraded products were known to have less herbicidal effect. The results confirms that the root growth of cucumber seedlings was relatively more in enzyme extract taken from transgenic lines, indicating that enzymes produced in the transgenic lines were capable of inactivating glyphosate after 3 hr of treatment.The mode of action of *igrA* and *GOX* are by detoxifying glyphosate whereas *EPSPS* alters the binding capacity to glyphosate, stacking these three genes into a single genetic background might enhance the glyphosate resistance.

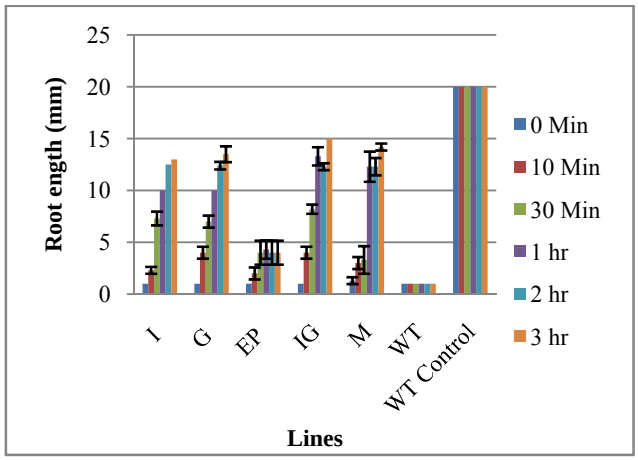

Fig. 2: Relative efficacy of crude enzyme extract of tobacco transgenic line in degrading glyphosate

5. ACKNOWLEDGEMENT

We are grateful to the DBT- HUB fellowship by Department of Biotechnology, Government of India, New Delhi, for generous financial assistance.

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