PROCEEDINGS OF THE BALKAN SCIENTIFIC CONFERENCE OF BIOLOGY IN PLOVDIV (BULGARIA) FROM 19TH TILL 21ST OF MAY 2005 (EDS B. GRUEV, M. NIKOLOVA AND A. DONEV), 2005 (P. 238–245)

GLUTAMINE SYNTHETASE AS A SIMPLE METHOD FOR DETECTION OF *BAR* GENE IN TRANSGENIC BARLEY LINES

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ABSTRACT. The enzyme glutamine synthetase allows the plant cell to convert ammonia, a product of various plant processes, into amino acids. Glutamine synthetase inhibitors block the activity of this enzyme. Glutamine synthetase activity (GS) was used for a possible *bar* gene detection. Transgenic barley plants were used as a model system to investigate the efficiency and simplicity of this method. Applying the method more of the tested transgenic plants determined by PCR and Western blotting analyses expressed higher glutamine synthetase activity. In this study we demonstrate the successful application of GS as a simple and effective indirect method *bar* gene monitoring.

KEY WORDS. *bar*, barley, glutamine synthetase, herbicide resistance
Abbreviations: GS - glutamine synthetase; MDA - malondyaldehyde; PPT – phosphinothricin;

INTRODUCTION

Bar gene of *Streptomyces hygroscopicus* encoding phosphinothricin acetyltransferase and conferrring bialaphos resistance have been widely used in plant genetic engineering: (1) as a selectable marker gene for transformation, (2) as a reporter gene in chimeric gene constructs, and (3) as dominant gene for engineering weed control in crop species (De Block et al., 1987; Thompson et al., 1987; White et al., 1990). Resistance to the broad-spectrum herbicides based on phosphinothricin (L-PPT) as the active compound has been obtained by introducing *bar* or *pat* gene into the plant. The chemically synthesized racemic mixture D,L-PPT is also known as

glufosinate. *Bar* gene is indeed difficult to detect reliably with PCR, and it seemed that every time an effective protocol and primers were passed to another researcher, the conditions had to be re-optimized (Bregitzer, personal communications). However, a sensitive and reliable PCR-based assay for the presence of *bar* would be highly desirable for rapid screening of putatively transformed plants. In a number of laboratories throughout the world, difficulties have been experienced in achieving bar amplification, possibly due to the high GC content (68.3%) of this gene. Long continuous GC sequences are present, including two closely adjacent stretches of 12 and 13 GC base pairs (Vickers et al., 1996). For confirmation the *bar* gene presence in putative transgenic barley plants many direct and indirect techniques were applied: leaves paintbrush with herbicide, PCR assay, Western and Southern blots, ELISA (STEFFENS Biotechnisch Analysen GmbH); thin layer chromatography (Trait LL leaf/ seed Test Kit, SDI – USA). Almost of these techniques are expensive and/or labour consuming.

Glutamine synthetase (GS) is found in all organisms owing to its crucial role in accumulation of NH_4^+ in bacteria as well as the participation of this enzyme in the amino acids metabolism in mammals by converting the toxic NH_4^+ in glutamine. Because of importance of GS much attention has been focused on this enzyme. L-PPT acts as a mimic of L-glutamate and inhibits GS resulting in ammonia accumulation and impairment of photosynthesis in plants (Wehrmann et al., 1996). Damage of cell membranes and inhibition of photosynthesis are followed by plant cell death. The action of glufosinate is dependent on environmental conditions. Temperatures below 10°C, as well as drought stress, reduce its efficacy because of the limited metabolic activity of the plant (Donn, 1982). Also, light is an important factor for the action of glufosinate (Koecher, 1983).

Glufosinate is not metabolized by treated plants. However, different metabolites of glufosinate are detected in plants transformed with the *pat* gene, than those found in plants not containing the gene. Three metabolites (4-methylphosphinico-2-oxobutanoic acid, 3-methylphosphinico-prpanoic acid, and 4-methylphosphinico-2-hydroxy-butanoic acid) were found in the non-transformed plant versus mainly one metabolite, the acetylated glufosinate, found in transformed plants. The three metabolites found in non-transformed plants were not detected in the transformed plants. This indicates that the transgene acetylation of glufosinate competes with plant specific pathway of glufosinate metabolism (Dröge-Laser et al., 1994; Dröge et al., 1992).

Using transgenic barley plants as a monocot experimental system we applied different attempts to identify *bar* gene: glutamine synthetase activity (Wallsgrove et al., 1979), malondyaldehyde (MDA) levels (Heath and Packer, 1968) and the content of plastid pigments (Arnon, 1949). In this study we demonstrate that the enzymatic activity could be used as reliable and simple method for proving of the transgene in plants showing tolerance to the herbicide.

MATERIAL AND METHODS

Plant material

Non-transformed and T2 generation from transformed with *bar* gene (construct pDM302) barley plants from Bulgarian cultivar Ruen were used. 12 transgenic lines were derived from 40 independent transformation events produced in AgroBioInstitute, Sofia using home-made gene gun (Abumhadi et al., 2001). Transgene integration and expression were confirmed by analyses based on PCR and Western blot as well as leaves paintbrush with herbicide.

In order to avoid age heterogeneity in the untransformed plants, two lines of control plants were used in all the experiments. One line was randomly selected from a population of untransformed 45 days-old barley plants and the other one two weeks later (60 days-old).

MDA assay

A 0.3-g leaf tissue sample was homogenized in 3 ml 0.1 % TCA on ice. After centrifugation at 10 000 g for 20 min supernatant was used for the assay. MDA was determined according to Dhindsa et al (1981), including TCA (trichloroacetic acid)/TBA (thiobarbituric acid) addition and a heat/cool cycle. Absorption was read at 532 nm and 600 nm. MDA concentration was calculated using its extinction coefficient 155 $mM^{-1}.cm^{-1}$ (Heath R.L. and Packer L.1968).

Plastid pigments

The content of plastid pigments (chlorophyll and carotenoids) of algal cells was determined spectrophotometrically according to Arnon (1949). Cells were harvested by centrifugation, sonicated and extracted with 80% (v/v) acetone at 4°C.

Glutamine synthetase activity

Glutamine synthetase activity was determined spectrophotometrically. A 1-g leaf tissue sample was homogenized in 5 ml extraction buffer (containing 50 mmol Tris – HCl, pH 7.8; 10 mmol $MgSO_4 \times 7H_2O$; 10 mmol EDTA; 10 mmol Cys; 1% PVP and 0.1% ME) on ice, centrifuged at 14000g for 45 min and supernatant was used for the assay according to the method of Wallsgrove et al. (1979).

For statistical significance the data were processed and assessed by LSD at a 5% level of probability.

RESULTS AND DISCUSSION

In genetically modified glufosinate-tolerant plants, the L-isomer of glufosinate is rapidly metabolized by the action of the enzyme phosphinothricin acetyltransferase (PAT) into the non-phytotoxic stable metabolite N-acetyl-L-glufosinate (2-acetamido-4-methylphosphinico-butanoic acid). N-acetyl-L-glufosinate does not inhibit glutamine synthetase. Therefore, no phytotoxic physiological effects are observed in genetically modified glufosinate-tolerant plants.

The inhibition of GS in plants is manifested by ammonia accumulation, inhibition of amino acid synthesis, and severe damage to plant tissues which ultimately result in death of the treated plant. At the final stage cell death involves membrane destruction and metabolites such as MDA can give an indication for membrane condition.

Glutamine synthetase activity

Forty five and sixty days-old transformed T2 plants were used for determination of the GS activity. For this purpose 12 transgenic barley plants and 2 sensitive plants were investigated. The 45 days-old transgenic plants had a 27 - 112% significant increase (LSD = 13.31) in the total leaf GS activity over the control plant (C1). Comparison of the transformed and untransformed plants showed that there was a clear increase in the level of total GS protein in the leaves of the transgenic T2 plants (Fig. 1). Highest activity of the enzyme (approximately two times greater than that of the control) was observed in lines 22, 26, 1 and 44 indicating high level of expression of *bar*. On the other hand, the 60 days-old plants showed a 16-25 % increase of the GS activity (Fig. 1) over the control (C2). The differences in GS activity values were caused by the variation in the expression of *bar* gene in the transgenic plants.

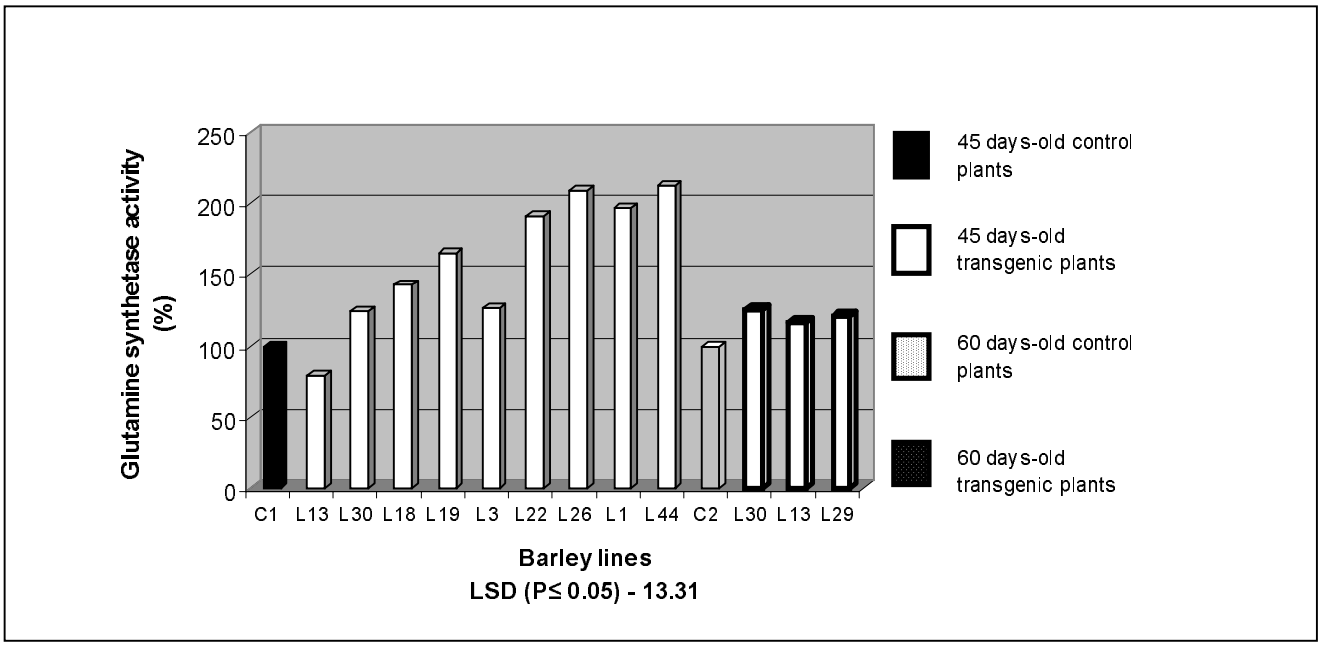

Figure 1. *Activity of glutamine synthetase in transgenic barley lines*

More-recent studies have shown that in the higher plants GS is encoded by a multigene family which is expressed in an organ-specific manner. Moreover, a species-specific pattern of gene expression could be observed according to the physiological status or the developmental stage of the plant. The specific regulatory mechanisms controlling transcription and translation of particular GS isoforms in different cellular compartments are still not fully understood. Altering ammonium

assimilation via genetic manipulation should provide more information about the organization and regulation of this complex enzyme (Vincent et al., 1997).

MDA assay

MDA is a widely used stress indicator of plant membrane damage and is a product of peroxidation of polyunsaturated fatty acids (Krammer et al., 1991). The products of lipid peroxidation (MDA) are found to be an indicator for the membranes state changes in different physiological stages as well as reports the influence of the stress factors. The amount of MDA for 45 days-old plants did not exceed that of the control ones showing that no membrane damages were occurred (Fig. 2) and the state of the tested plants was normal. These results indicated that transgenic plants could be used as a model system for study on the physiological status of the membranes before and after application of the herbicide. In this study we investigated that all the tested transgenic plant show low amount of MDA in 45 days old plants – 8-45% significant decrease (LSD= 8.21). The higher content of MDA in 60 days-old plants was varied from 29 to 41 % (Fig.2). These observations are in agreement with the results of Vincent et al. (1997) where they are explained that expression of GS in the plants may accelerate plant development, leading to early senescence.

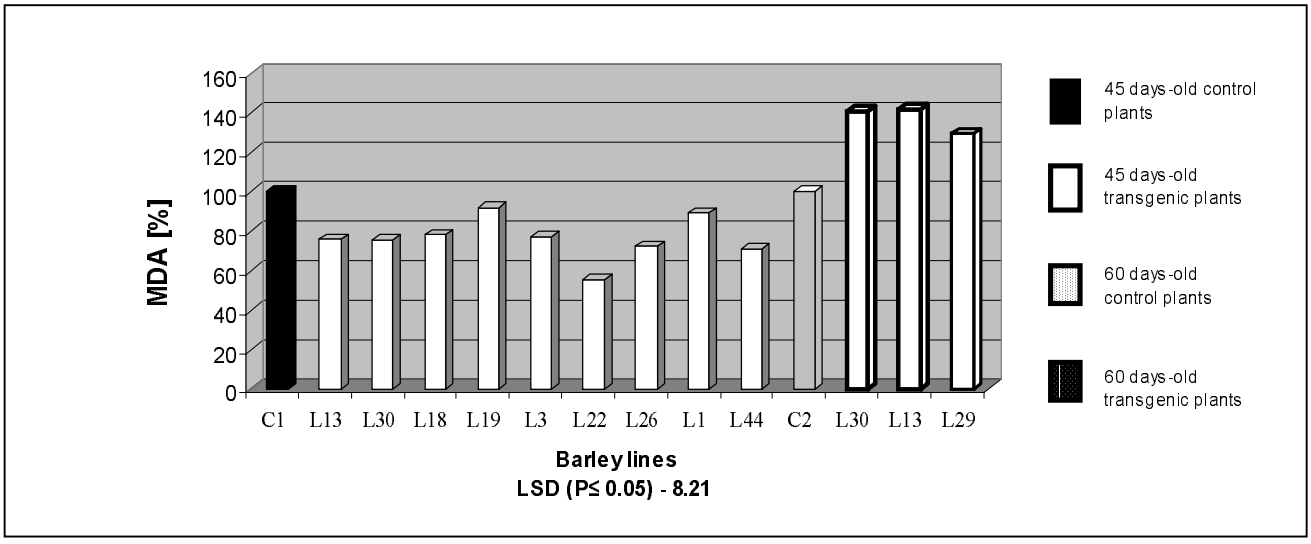

Figure 2. *Content of malondyaldehyde in transgenic barley lines*

Plastid pigments

Both enzymatic and not-enzymatic antioxidants (phenols, alkaloids, amins, vitamins, amino acids, carotinoids, etc) act in the plant cells. Several natural compounds as thyols, ascorbic acid, tannin, cofein, flavonoids, and carotinods may protect the cell against biotic and abiotic agents and play a fundamental role in antimutagenesis processes. The photoprotect function of carotinoids plays a crucial role in all of the photosynthesizing organisms. Carotinoids are able to deactivate the singlet oxygen and to saturate the triplet form of the chlorophyll resulting in reducing

of the active oxygen release (Siefermann-Harms, 1987). The effective protect against UV waves depends on both the synthesized carotinoids and its accumulated amount.

In our study we determined the amount of carotinoids in transgenic and control plant. The majority of the tested lines had a higher amount of carotinoids compared to control non-transformed lines (Fig. 3). The results showed a increase of 30-55 % in 45 days-old plants and 15-18% in 60 days-old plants (LSD = 19.95). Similar results were observed in transformed with *bar* gene and non-transformed tobacco plants. The amount of carotinoids in transgenic tobacco plants was higher comparing with non-transformed plants before and after herbicide application (unpublished data).

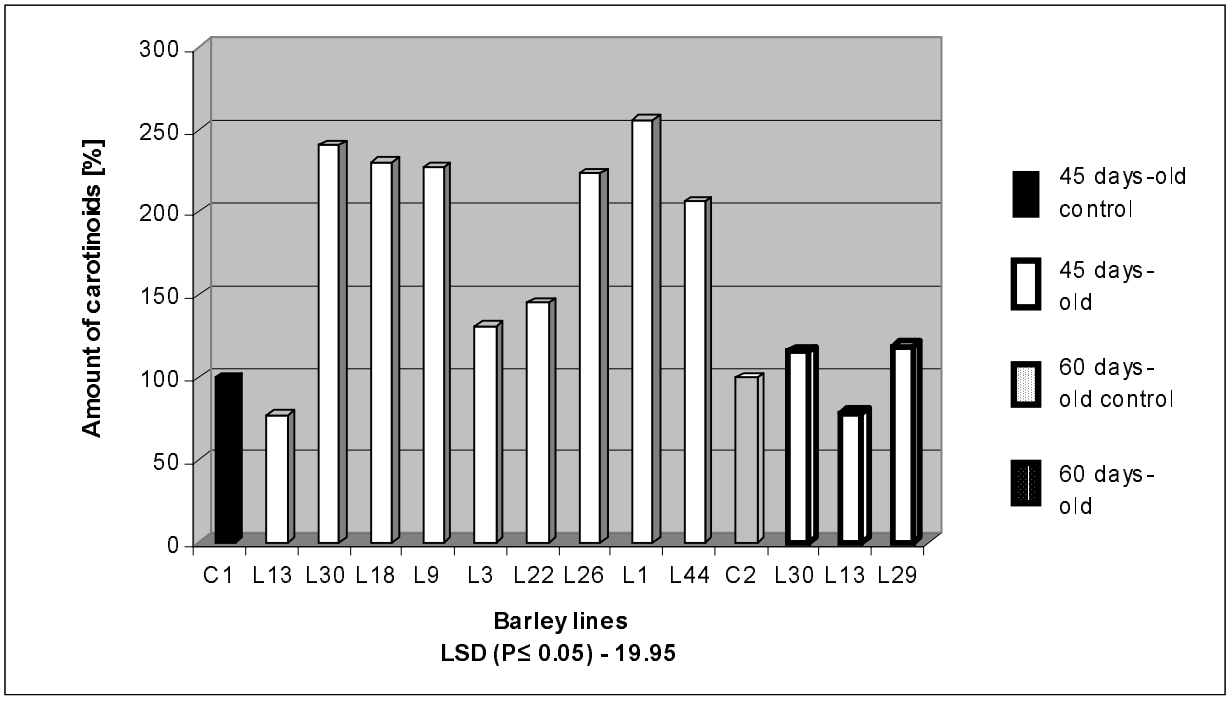

Figure 3. *Amount of car in transgenic barley lines*

Herbicide bioassay

Currently seed companies are developing crop plants which are tolerant to different herbicides. Modern herbicide research aims at the development of products which meet the farmer's requirements and have the lowest possible side effects. As a result, more environmentally acceptable herbicides are available. *Bar* and *pat* genes have been used successfully in transferring herbicide resistance to glufosinate in a number of crop plants.

Herbicide sensitivity can be checked by either spraying the entire plant with 0.5% (v/v) Liberty® solution plus 0.1% Tween 20 on plants at 45 and 60 days stage or by painting a section of leaf blade (Wan and Lemaux, 1994). The treated plants were examined after one week. Plants expressing *bar* gene showed no symptoms. Non-transformed plants or plants not expressing *bar* will not show several leaf necrosis, or die.

CONCLUSIONS

Glutamine synthetase activity, malondyaldehyde levels and the content of plastid pigments is an efficient system for studying herbicide tolerance in plants when are exposed to the herbicide and, after a period of growth time regarding to their rate as tolerant or susceptible. Whereas PCR looks for presence of the desired DNA and an ELISA finds the protein coded for by that DNA, the bioassay determines the actual effect or activity of the gene in a living plant.

Testing for herbicide tolerance in transgenic barley plants by GS is a simple and effective method for confirming transformation and the continued expression of *bar* transgene across the generation.

ACKNOWLEDGMENTS

This study was supported by Sofia University Foundation for Scientific Investigations (Project No 103/2004).

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