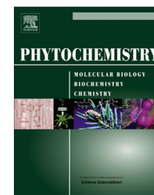
Phytochemistry 96 (2013) 117–122

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem



Liquid chromatography–diode array detection to study the metabolism of glufosinate in *Triticum aestivum* T-590 and influence of the genetic modification on its resistance

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ARTICLE INFO

Article history:
Received 20 March 2013
Accepted 10 October 2013
Available online 1 November 2013

Keywords:
Triticum aestivum
GMO crops
Glufosinate
Resistance
Metabolism
Degradation
LC–DAD
LC–TOF/MS

ABSTRACT

The resistance to glufosinate of two lines—genetically modified (GM) and unmodified (T-590 and T-549, respectively)—of *Triticum aestivum* has been studied. In the GM line, the *bar* gene was introduced to increase the resistance to glufosinate. Experiments in a controlled growth chamber showed that line T-590 presented a high resistance to glufosinate with an ED_{50} value of 478.59 g active ingredient per hectare (g ai ha^{-1}) *versus* 32.65 g ai ha^{-1} for line T-549. The activity of glutamine synthetase (GS) in leaf extracts from both lines was investigated. The I_{50} for line T-590 was 694.10 μM glufosinate *versus* 55.46 μM for line T-549, with a resistance factor of 12.51. Metabolism studies showed a higher and faster penetration of glufosinate in line T-549 than in line T-590. LC–TOF/MS analysis of glufosinate metabolism at 48 h after herbicide treatment (300 g ai ha^{-1}) revealed an 83.4% conversion of the herbicide (66.5% in *N*-acetyl-glufosinate metabolite), while in line T-549 conversion of the herbicide was about 40% (0% to *N*-acetyl-glufosinate). These results suggest that metabolism of glufosinate by the *bar* gene is a key mechanism of resistance in line T-590 that explains such high levels of herbicide tolerated by the plant, together with other mechanisms due to unmodified pathway, absorption and loss of glufosinate affinity for its target site.

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1. Introduction

The amino acid isolated in 1972 in a German laboratory from one species of the soil bacterium *Streptomyces viridochromogenes* was butanoic acid, L-2-amino-4-(hydroxymethylphosphinyl)-, to which the name of phosphinothricin was given by Bayer (Bayer et al., 1972; Hoerlein, 1994; OECD, 1999). Later, this compound showed herbicidal activity and received the name of glufosinate ammonium, which is the active ingredient in the commercial herbicide all over the world. Glufosinate inhibits the activity of the enzyme glutamine synthetase (GS) being competitively fixed in the substrate (glutamate) binding site (Bayer et al., 1972; Lea et al., 1984; Hoagland, 1999). In this way the synthesis of L-glutamine is inhibited, thus decreasing the levels of aspartic acid, asparagine and other amino acids which are precursors in the chemical synthesis of nucleic acids and proteins. The synthesis of L-glutamine is also a mechanism for incorporating ammonia (NH_3) in plants (Hoerlein, 1994). Treatment with glufosinate causes accumulation of ammonia (Tachibana et al., 1986) and decreases photosynthesis (Sauer et al., 1987).

The advances in genetic engineering have promoted the application of recombinant DNA technology to obtain and commercialize new varieties of genetically modified crops with preset properties which offer significant advantages to farmers who grow them, facilitating, among other things, a better control of weeds.

The introduction of a gene to produce herbicide resistance in plants can be achieved by two approaches. The first consists of inserting into the plant the gene of an enzyme which is not inhibited by the herbicide in which glyphosate resistance is based; in the second approach, the inserted gene corresponds to an enzyme that modifies the herbicide in which the mechanism of glufosinate resistance is based.

At the end of the 80s of the past century, two glufosinate resistance genes were identified and designed as *pat* and *bar*, isolated from different *Streptomyces* species, both encode one of the phosphinothricin acetyltransferases (PAT), widely applied in plant genetic engineering. The structural and functional equivalence of the PAT proteins is shown by the similar performance of transgenic plants carrying the *bar* or *pat* gene (Wehrmann et al., 1996). The

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0031-9422/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.phytochem.2013.10.008

identification of the resistance of *Streptomyces* to glufosinate suggested that these bacteria had a biochemical mechanism to maintain endogenous GS activity. PAT enzyme consists of 183 amino acids (Wehrmann et al., 1996) acetylated to phosphinothricin (glufosinate) in the N-terminus. The generated product, butanoic acid, 2-(acetylamino)-4-(hydroxymethylphosphinyl)-, known as *N*-acetyl-glufosinate, does not present herbicidal activity (Thompson et al., 1987; Wehrmann et al., 1996; Wohlleben et al., 1988; Broer et al., 1989; Dröge et al., 1992).

The movement of transgenes to wild relatives takes place by pollen, and the production of viable hybrids depends on the physical proximity and synchrony of flowering in GM plants with sexually compatible species. However, the introgression of glufosinate tolerance in sexually compatible weed populations is possible and has the potential to cause control problems (Mallory-Smith and Zapiola, 2008; Warwick et al., 2008).

The metabolism of glufosinate has been extensively studied both in soil (Tebbe and Reber, 1988, 1991; Smith, 1988, 1989; Behrendt et al., 1990; Gallina and Stephenson, 1992) and plants (Haas, 1986; Dröge et al., 1992, 1994) using a large number of analytical techniques for separation and identification of such metabolites. These studies showed that glufosinate in soil is rapidly transformed into butanoic acid, 4-(hydroxymethylphosphinyl)-2-oxo-(PPO), which becomes propanoic acid, 3-(hydroxymethylphosphinyl)-(MPP) by decarboxylation; then, PPO suffers a reduction with lost of oxygen in position 2 and formation of butanoic acid, 4-(hydroxymethylphosphinyl)-(MPB) (Behrendt et al., 1990).

The metabolism of glufosinate has also been studied both in unmodified plants that presented natural resistance, and in GM plants. These studies showed that the metabolites described in soil also appear in unmodified plants (Dröge et al., 1992, 1994) in addition to the known metabolite butanoic acid, 2-hydroxy-4-(hydroxymethylphosphinyl)-(MHB) (Dröge et al., 1994). The studies were performed on crops as soybean (*Glycine max*), wheat (*Triticum aestivum*), and maize (*Zea mays*) (Komossa and Sandermann, 1992) that presented even some tolerance to glufosinate. In addition to the above metabolites, the compound resulting from acetylation of glufosinate by the PAT enzyme (*N*-acetylphosphinothricine), which produces a rapid decrease in the content of glufosinate in the plant, was found in GM plants. This and the other metabolites are transported to the top of the plant, as demonstrated by Dröge et al. (1994) and Beriault et al. (1999).

The economic importance of wheat at the global level results from its use for a large number of processed foods for humans and animals. This importance relies on the viscoelastic properties of wheat flour dough, which allow wheat to be used for making bread and many other food products such as cake, biscuits, pasta or noodles. The transgenic line T-590 studied in this work showed an improved breadmaking quality as a consequence of the expression of the 1Dy10 high molecular weight glutenin subunit gene (León et al., 2009). Glufosinate resistance was incorporated in this line along with the 1Dy10 gene. Despite glufosinate is an important tool for weeds control, proper function in wheat requires genetic modification of this crop.

The aim of this research was to study the effectiveness of introducing the *bar* gene in wheat as compared to the unmodified genotype, and to show the relative importance of the involved pathways using two lines of the same genotype of wheat, an unmodified line and other engineered with *bar* gene. The unavailability of some of the metabolites led to plan identification of them by liquid chromatography–time-of-flight mass spectrometry (LC–TOF/MS) for subsequent determination of the precursor and metabolites by cheaper and easier to handle equipment: a liquid chromatography coupled to a diode array detector (LC–DAD).

2. Results and discussion

2.1. Dose–response assays

The shoot biomass (stems and leaves) production in both T-549 and T-590 wheat lines was reduced by application of glufosinate.

The ED_{50} (herbicide concentration required for a 50% reduction of plants fresh weight) for line T-590 was 478.59 grams of active ingredient per hectare (g ai ha^{-1}) *versus* 32.65 g ai ha^{-1} for line T-549 (Table 1). These results, obtained from the whole plant dose–response bioassay, show that the line T-590 was 14.66 times more resistant than line T-549; therefore, the former is highly resistant to glufosinate. Compared with the values given for ryegrass (Ávila-García and Mallory-Smith, 2011) and goosegrass (Jalaludin et al., 2010; and Seng et al., 2010), the resistance factor of this wheat is much higher, and the ED_{50} was similar to the GM described by Ávila-García et al. (2012), thus offering strong competition with these weeds.

2.2. Glutamine synthetase activity assays

The specific *in vitro* activity of GS obtained from shoot tissue of lines T-549 and T-590 was similar (364.68 ± 29.21 and 378.14 ± 15.07 nmol of glutamine mg^{-1} of protein h^{-1}, respectively). Line T-549 presented an I_{50} = 55.46 μM *versus* T-590 with an I_{50} = 694.10 μM. The resistance factor for *in vitro* glufosinate was 12.52 (Table 2). These results suggest that the resistance to glufosinate in the GM population is conferred by an altered GS enzyme. In this case the I_{50} for the GM wheat was lower than that for the GM population described by Ávila-García et al. (2012). The selected glufosinate-resistant cells required between 2.6 and 4.5 times greater glufosinate concentration than the susceptible cells to inhibit the GS enzyme activity by 50%, thus indicating that an altered target site was responsible for resistance.

2.3. Glufosinate metabolism

Extracts from T-549 and T-590 wheat leaves were injected into both the LC–TOF/MS and LC–DAD arrangements for separation–identification and separation–quantification, respectively. As shows Fig. 1, the metabolites were correctly identified in line T-590 as glufosinate, PPO, MHB, MPP, MPB and *N*-acetyl-glufosinate. The precursor ions at *m/z* 402.1112, 420.1218, 151.0166, 179.0115, 181.0271 and 165.0322, corresponded to $[M-H]^+$ adducts with theoretical formula $C_{20}H_{22}NO_6P$, $C_{22}H_{24}NO_7P$, $C_4H_9O_4P$, $C_5H_9O_5P$, $C_5H_{11}O_5P$ and $C_5H_{11}O_4P$, respectively. These peaks were identified as 2-amino-4-(hydroxymethylphosphinyl)-butanoic acid (glufosinate), 2-(acetylamino)-4-(hydroxymethylphosphinyl)-butanoic acid (*N*-acetyl-glufosinate), 3-(hydroxymethylphosphinyl)-propanoic acid (MPP), 4-(hydroxymethylphosphinyl)-2-oxo-butanoic acid (PPO), 2-hydroxy-4-(hydroxymethylphosphinyl)-butanoic acid (MHB) and 4-(hydroxymethylphosphinyl)-butanoic acid (MPB), the structures of which appear in Fig. 2.

The retention times of the target compounds were 9.046, 5.299, 2.624, 8.680, 3.661 and 3.137 min for glufosinate, *N*-acetyl-glufosinate, MPP, PPO, MHB and MPP, respectively, which were used in the experiments developed by LC–DAD equipment. The results obtained in this case show clear differences between lines with respect to the exposure time and doses used (Table 3). Penetration of the herbicide was higher and faster in T-590 wheat plants (Table 3). Regarding metabolism, line T-590 undergone metabolism (at 6 h after treatment) by the two pathways of glufosinate degradation described by Dröge et al. (1994) in contrast to line T-549, which presents only the natural pathway described in soils (at 24 h after treatment). Metabolism appeared at medium and high

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